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DescriptionTechnical Field

5 The present invention relates to blood coagulation factors in general, and more specifically, to the expression of proteins having biological activity for blood coagulation.

Background Art

10 Blood coagulation is a process consisting of a complex interaction of various blood components or factors which eventually gives rise to a fibrin clot. Generally, the blood components which participate in what has been referred to as the coagulation "cascade" are proenzymes or zymogens, enzymatically inactive proteins which are converted to proteolytic enzymes by the action of an activator, itself an activated clotting factor. Coagulation factors which have undergone such a conversion are generally referred to as
 15 "activated factors," and are designated by the addition of a lower case postscript "a" (e.g., VIIa).

There are two separate systems which can promote blood clotting and thereby participate in normal haemostasis. These systems have been referred to as the intrinsic and the extrinsic coagulation pathways. The intrinsic pathway refers to those reactions which lead to thrombin formation through utilization of factors present only in plasma. An intermediate event in the intrinsic pathway is the activation of Factor IX to Factor IXa, a reaction catalyzed by Factor XIa and calcium ions. Factor IXa then participates in the activation of
 20 Factor X in the presence of Factor VIIIa, phospholipid and calcium ions. The extrinsic pathway involves plasma factors as well as components present in tissue extracts. Factor VII, one of the proenzymes referred to above, participates in the extrinsic pathway of blood coagulation by converting (upon its activation to VIIa) Factor X to Xa in the presence of tissue factor and calcium ions. Factor Xa in turn then converts
 25 prothrombin to thrombin in the presence of Factor Va, calcium ions and phospholipid. Because the activation of Factor X to Factor Xa is an event shared by both the intrinsic and extrinsic pathways, Factor VIIa can be used for the treatment of patients with deficiencies or inhibitors of Factor VIII (Thomas, U. S. Patent 4,382,083). There is also some evidence to suggest that Factor VIIa may participate in the intrinsic pathway as well (Zur and Nemerson, *J. Biol. Chem.* 253: 2203-2209, 1978) by playing a role in the
 30 activation of Factor IX.

Experimental analysis has revealed that human Factor VII is a single-chain glycoprotein with a molecular weight of approximately 50,000 daltons. In this form, the factor circulates in the blood as an inactive zymogen. Activation of Factor VII to VIIa may be catalyzed by several different plasma proteases, such as Factor XIIa. Activation of Factor VII results in the formation of two polypeptide chains, a heavy
 35 chain ($M_r = 28,000$) and a light chain ($M_r = 17,000$), held together by at least one disulfide bond. Factor VII may also be activated to VIIa in vitro, for example, by the method disclosed by Thomas in U.S. Patent No. 4,456,591.

Factor IX circulates in the blood as a single-chain precursor of molecular weight 57,000 and is converted to an active serine protease (Factor IXa) upon cleavage by Factor XIa. Factor IXa consists of a
 40 light chain and a heavy chain of molecular weights 16,000 and 29,000, respectively.

Current treatment practices for patients having coagulation disorders (e.g., deficiencies of Factor VIII and IX) generally involve replacement therapy with cryoprecipitate or other fractions of human plasma containing enriched levels of a particular factor. These preparations have heretofore been obtained from pooled human plasma, although the preparation of cryoprecipitates requires the use of a relatively large
 45 amount of human plasma as starting material.

Therapeutic uses of Factor VII exist in the treatment of individuals exhibiting a deficiency in Factor VII, as well as Factor VIII and Factor IX deficient populations, and individuals with Von Willebrand's disease. More specifically, individuals receiving Factors VIII and IX in replacement therapy frequently develop antibodies to these proteins. Continuing treatment is exceedingly difficult because of the presence of these
 50 antibodies. Patients experiencing this problem are normally treated with an activated prothrombin complex known to consist of a mixture of active and inactive clotting enzymes, including Factor VIIa. Further, recent studies indicate that small amounts (40-50 micrograms) of injected Factor VIIa are effective in controlling serious on-going bleeding episodes in Factor VIII deficient patients who have high levels of antibody in their blood (Hedner and Kisiel, *J. Clin. Invest.* 71: 1836-1841, 1983).

55 Due to the diverse sources of the plasma used in the preparation of cryoprecipitates, it is difficult to test the preparations to ensure that they are free of viral contamination. For instance, essentially all recipients of cryoprecipitate show a positive test for hepatitis. Recent reports have also indicated that some hemophiliacs receiving cryoprecipitate have developed acquired immune deficiency syndrome (AIDS). In addition, the

purification of large amounts of these factors is extremely difficult and expensive.

Consequently, there exists a need in the art for a method of producing relatively large quantities of pure preparations of Factor VIIa and Factor IX. The present invention fulfills this need through the use of recombinant DNA technology, successfully eliminating the problem of viral contamination and, at the same time, providing a consistent and homogenous source of active Factor VIIa to treat Factor VII and Factor IX deficient patients and individuals with Von Willebrand's disease.

Disclosure of the Invention

Briefly stated, the present invention discloses a DNA construct containing a nucleotide sequence encoding human factor VII having an amino acid sequence as shown in Figure 1b. The nucleotide sequence may comprise a first nucleotide sequence derived from a cDNA or a genomic clone of Factor VII joined to a second nucleotide sequence positioned downstream of the first sequence, said second nucleotide sequence derived from a cDNA clone of of Factor VII. The joined sequences code for a protein which upon activation has Factor VII a biological activity for blood coagulation. Further, the first nucleotide sequence may also encode a leader peptide and may also include a double-stranded oligonucleotide.

In addition, the present invention discloses recombinant plasmids capable of integration in mammalian host cell DNA. One of the plasmids includes a promoter followed downstream by a set of RNA splice sites, the RNA splice sites being followed downstream by a nucleotide sequence which codes for Factor VII. The sequence codes for a protein which upon activation has Factor VIIa biological activity for blood coagulation. The nucleotide sequence is then followed downstream by a polyadenylation signal.

Similar to the recombinant plasmid noted above, the present invention also discloses a second plasmid which includes a promoter followed downstream by a set of RNA splice sites, the RNA splice sites being followed downstream by a nucleotide sequence which codes at least partially for Factor IX. The nucleotide sequence comprises a first nucleotide sequence which encodes a calcium binding domain joined to a second nucleotide sequence positioned downstream of the first sequence. The second nucleotide sequence encodes a catalytic domain for the serine protease activity of Factor IX. The joined sequences code for a protein having substantially the same biological activity for blood coagulation as Factor IX. The nucleotide sequence is then followed downstream by a polyadenylation signal.

A third aspect of the invention discloses mammalian cells stably transfected to produce a protein having substantially the same biological activity, upon activation, as Factor VIIa. The cells are transfected with a DNA construct containing a nucleotide sequence which codes for Factor VII. The sequence codes for a protein which, upon activation, has Factor VIIa biological activity for blood coagulation.

The present invention further provides for a method of producing a protein having biological activity for blood coagulation mediated by Factor VIIa through establishing a mammalian host cell which contains a DNA construct containing a nucleotide sequence which codes for Factor VII. The sequence codes for a protein which, upon activation, has Factor VIIa biological activity for blood coagulation. Subsequently, the mammalian host is grown in an appropriate medium containing vitamin K and the protein product encoded by the DNA construct and produced by the mammalian host cell is isolated. The protein product is then activated to generate Factor VIIa.

Yet another aspect of the present invention discloses a DNA construct comprising a DNA sequence encoding Factor VII having an amino acid sequence as shown in Figure 1b. In a preferred embodiment, the DNA sequence comprises the cDNA sequence of Figure 1b from bp 36 to bp 1433. In another preferred embodiment, the DNA sequence comprises the cDNA sequence of Figure 1b from bp 36 to bp 99, followed downstream by the sequence from bp 166 to bp 1433. Recombinant plasmids capable of integration in mammalian host cell DNA comprising the DNA sequences described immediately above are also disclosed.

Mammalian cells stably transfected with a recombinant plasmid comprising a DNA sequence encoding Factor VII having an amino acid sequence as shown in Figure 1b are also disclosed. In preferred embodiments, the DNA sequence comprises the cDNA sequence of Figure 1b from bp 36 to bp 1433, or the cDNA sequence of Figure 1b, from bp 36 to bp 99, followed downstream by the sequence from bp 166 to bp 1433.

A method for producing a protein having biological activity for blood coagulation mediated by Factor VIIa through establishing a mammalian host cell that contains a DNA construct as described above is also disclosed. The mammalian host cell is subsequently grown in an appropriate medium containing vitamin K, and the protein product encoded by the DNA construct is isolated. The protein product is then activated to generate Factor VIIa.

Other aspects of the invention will become evident upon reference to the following detailed description and attached drawings.

Brief Description of the Drawings

Figure 1a illustrates the partial Factor VII cDNA sequence produced by joining portions of cDNA clones λVII2115 and λVII1923.

5 Figure 1b illustrates the Factor VII cDNA sequence of λVII2463. Arrows indicate the extent of the deletion in the sequence of λVII565. Numbers above the sequence designate amino acids. Numbers below designate nucleotides.

Figure 2a illustrates the amino acid sequences of the amino terminal regions of several clotting factors.

10 Figure 2b illustrates a comparison of the amino acid sequence of Factor VII obtained from protein sequencing with that encoded by the cDNA.

Figure 3 illustrates the joining of Factor IX leader sequences to a sequence encoding a consensus calcium binding domain.

Figure 4 illustrates the joining of the Factor IX-consensus sequence hybrids to a partial Factor VII cDNA to produce an in-frame coding sequence.

15 Figure 5 illustrates the construction of a plasmid containing a coding sequence for a Factor IX/Factor VII fusion protein.

Figure 6 illustrates the expression vector FIX/VII/pD2. Symbols used are Ad2 MLP, the major late promoter from adenovirus 2; L1-3, the adenovirus 2 tripartite leader sequence; 5'ss, 5' splice site' 3'ss, 3' splice site' and pA, the late polyadenylation signal from SV40.

20 Figure 7 illustrates the nucleotide sequence of a Factor IX/Factor VII cDNA fusion.

Figure 8 illustrates expression vector pM7135. Symbols used are E, the SV40 enhancer; ori, the 0-1 map units Ad 5; pA, the early polyadenylation signal from SV40; Δ, the deletion region of the pBR322 "poison" sequences; and other symbols as described for Figure 6.

Figure 9 illustrates the subcloning of the 2463bp Factor VII cDNA.

25 Figure 10 illustrates the subcloning of the 565bp Factor VII cDNA.

Figure 11 illustrates the joining of the 5' end of pVII565 and the 3' portion of pVII2463in pUC18 to generate pVII2397.

30 Figure 12 illustrates the construction of the expression plasmids FVII(2463)/pDX and FVII(565+2463)/pDX. pA denotes the polyadenylation signal from SV40 in early or late orientation, as described in Example 9. Other symbols are as described for Figure 8.

Best Mode for Carrying Out the Invention

35 Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

Complementary DNA or cDNA: A DNA molecule or sequence which has been enzymatically synthesized from the sequences present in a mRNA template.

40 DNA Construct: A DNA molecule, or a clone of such a molecule, either single- or double-stranded, which may be isolated in partial form from a naturally occurring gene or which has been modified to contain segments of DNA which are combined and juxtaposed in a manner which would not otherwise exist in nature.

Plasmid or Vector: A DNA construct containing genetic information which may provide for its replication when inserted into a host cell. A plasmid generally contains at least one gene sequence to be expressed in the host cell, as well as sequences which facilitate such gene expression, including promoters and transcription initiation sites. It may be a linear or closed circular molecule.

45 Joined: DNA sequences are said to be joined when the 5' and 3' ends of one sequence are attached, by phosphodiester bonds, to the 3' and 5' ends, respectively, of an adjacent sequence. Joining may be achieved by such methods as ligation of blunt or cohesive termini, by synthesis of joined sequences through cDNA cloning, or by removal of intervening sequences through a process of directed mutagenesis.

50 Leader Peptide: An amino acid sequence which occurs at the amino terminus of some proteins and is generally cleaved from the protein during subsequent processing and secretion. Leader peptides comprise sequences directing the protein into the secretion pathway of the cell. As used herein, the term "leader peptide" may also mean a portion of the naturally occurring leader peptide.

Domain: A three-dimensional, self-assembling array of specific amino acids in a protein molecule which contains all or part of the structural elements necessary for some biological activity of that protein.

55 Biological Activity: A function or set of functions performed by a molecule in a biological context (i.e., in an organism or an *in vitro* facsimile). Biological activities of proteins may be divided into catalytic and effector activities. Catalytic activities of clotting factors generally involve the activation of other factors

through the specific cleavage of precursors. Effector activities include specific binding of the biologically active molecule to calcium or other small molecules, to macromolecules such as proteins, or to cells. Effector activity frequently augments, or is essential to, catalytic activity under physiological conditions. Catalytic and effector activities may, in some cases, reside within the same domain of a protein.

For Factor VIIa, biological activity is characterized by the mediation of blood coagulation through the extrinsic pathway. Factor VIIa activates Factor X to Factor Xa, which in turn converts prothrombin to thrombin, thereby initiating the formation of a fibrin clot. Because the activation of Factor X is common to both the extrinsic and intrinsic pathways of blood coagulation, Factor VIIa may be used to treat individuals severely deficient in the activities of Factor IX, Factor VIII or Von Willebrand Factor.

As noted above, the isolation of Factor VII from human plasma is a time-consuming and expensive process since the factor is a rare protein present only at a concentration of approximately 300 micrograms per liter of blood. In addition, it is difficult to separate from prothrombin, Factor IX and Factor X and is susceptible to proteolytic attack during purification (Kisiel and McMullen, *ibid*). Although single-chain human Factor VII has been purified to homogeneity (Kisiel and McMullen, *ibid*), the published purification methods are generally limited by low yield and/or contamination by other coagulation factors.

Factor VII is produced in the liver and requires vitamin K for its biosynthesis. Vitamin K is necessary for the formation of specific gamma-carboxyglutamic acid residues in the factors. These unusual amino acid residues, which are formed by a post-translational modification, bind to calcium ions and are responsible for the interaction of the protein with phospholipid vesicles. In addition, Factor VII contains one β -hydroxyaspartic acid residue which is also formed after the protein has been translated. However, the role of this amino acid residue is not known.

Given the fact that the activity of Factor VII is dependent upon post-translational modifications involving the gamma carboxylation of specific glutamic acid residues, and may also be dependent upon the hydroxylation of a specific aspartic acid residue, it is unlikely that an active product could be produced through the cloning and expression of Factor VII in a microorganism.

Accordingly, the present invention provides a method of producing a protein having biological activity for blood coagulation mediated by Factor VIIa using stably transfected mammalian cells.

As noted above, Factor VII requires vitamin K for its biosynthesis. In addition, the plasma proteins prothrombin, Factor IX, Factor X, Protein C, and Protein S also require vitamin K for their biosynthesis. The amino-terminal portions of these proteins, which contain gamma-carboxyglutamic acid residues, are homologous in both amino acid sequence and in biological function (Figure 2a). Further, the carboxy-terminal portions of Factor VII, prothrombin, Factor IX, Factor X, and Protein C determine their specific serine protease functions.

Factor VII is a trace plasma protein, and the mRNA encoding Factor VII is believed to be rare. Consequently, purification of Factor VII from plasma in sufficient quantities to permit extensive sequence analysis and characterization remains difficult. Degradation of Factor VII during purification, even in the presence of protease inhibitors, was noted by Kisiel and McMullen (*ibid*). Due to these difficulties, Factor VII has been poorly characterized, compared to other more abundant components of the blood coagulation system. Indeed, the work of Kisiel and McMullen (*ibid*) yielded sequence information for only 10 residues of each chain of Factor VII, and in each sequence the identification of two residues was tentative. Partial amino acid sequence data for Bovine Factor VII have also been published (DiScipio et al., *ibid*).

The presumed rarity of Factor VII mRNA has contributed to the lack of knowledge of the Factor VII gene. The success of conventional cDNA cloning techniques is dependent on a sufficient quantity of mRNA for use as a template. Premature termination of reverse transcription results in the production of cDNA clones lacking the 5' end and this condition is exacerbated by low mRNA levels. Several strategies for cDNA cloning of low abundance message have been developed (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982), but a lack of knowledge of the amino acid sequence of the product of interest makes it impossible to predict the DNA sequence and to design appropriate oligonucleotide probes. While it may be relatively straightforward to obtain a partial cDNA clone of a gene encoding a rare protein by using these advanced strategies, full-length cDNA clones of genes encoding rare proteins such as Factor VII remain exceedingly difficult to obtain.

In comparison to Factor VII, Factor IX is a relatively abundant protein and the sequence of a cDNA clone of the human Factor IX gene is known (Kurachi and Davie, *Proc. Natl. Acad. Sci. USA* 79: 6461-6464, 1982; and Anson et al., *EMBO J.* 3: 1053-1060, 1984). The structure of the Factor IX gene has been characterized and the amino acid sequence of the protein has been determined on the basis of the known nucleotide sequence. Some protein sequence data have also been published for human and bovine Factor IX and the sequences analyzed (DiScipio et al., *ibid*). The amino terminal portion of the protein contains 12 glutamic acid residues that are converted to γ -carboxyglutamic acid (Gla) residues in the mature protein.

The cleavage sites involved in the activation of Factor IX have also been identified (Kurachi and Davie, *ibid*). A sequence at the 5' end of the Factor IX cDNA clone codes for a signal peptide which is typical of those found in most secreted proteins (Kurachi and Davie, *ibid*). The expression of the Factor IX gene through recombinant DNA methods has not been previously reported.

5 Because of the difficulty in obtaining a full-length cDNA clone of the Factor VII gene, three novel approaches were adopted to supply the 5' end of the coding sequence, including the region encoding the leader peptide. According to the first method, a partial cDNA clone for Factor VII is joined to a fragment encoding the leader peptide and 5' portion of Factor IX. This approach is based on the observation that the amino-terminal portions of the two molecules are responsible for the calcium binding activities of the
10 respective proteins and the discovery that the calcium binding activity of Factor IX can substitute for that of Factor VII. The resultant polypeptide retains the biological activity of authentic Factor VII because the specific serine protease activities of the coagulation factors reside in the carboxy-terminal regions of the molecules. The second approach combines the partial cDNA clone with a DNA sequence encoding the leader and amino-terminal regions of Factor VII. The partial cDNA and amino acid sequences of Factor VII
15 disclosed herein enable the screening of a genomic DNA library or cDNA library for clones comprising the 5' portion of the Factor VII gene. The third approach involves joining the partial cDNA clone to hybrid coding sequences comprising a cDNA fragment encoding the leader peptide of Factor IX and a synthetic gene segment encoding a consensus calcium binding domain or a predicted amino terminal sequence for Factor VII. The coding sequence for the amino terminus of Factor VII was established through previously
20 unpublished amino acid sequence data disclosed herein. The consensus sequence was derived from the factor VII data and published sequence data for other vitamin K-dependent plasma proteins.

Consistent with the approach described above for screening for clones comprising the 5' portion of the Factor VII gene, the inventors have been successful in obtaining a full-length, correct cDNA that is suitable for expression.

25 Among the cDNA clones that were generated, a clone designated "λVII2463" contained the largest Factor VII cDNA insert. It was found to contain the entire coding sequence for Factor VII. This clone included a 35 nucleotide 5' untranslated region, 180 nucleotides coding for a 60 amino acid leader, 1218 nucleotides coding for the 406 amino acid mature protein, a stop codon, 1026 nucleotides of 3' untranslated sequence, and a 20 base poly(A) tail (beginning at position 2463). This cDNA has now been sequenced in
30 its entirety on both strands. A comparison of it with two cDNA inserts isolated earlier from clones λVII2115 and λVII1923, revealed that λVII2463 contains, on a single EcoRI fragment, a Factor VII cDNA coding for Factor VII leader and mature protein sequences.

A second clone, λVII565, was isolated that contained a cDNA insert that was identical to the cDNA of clone λVII2463 from nucleotide 9 to nucleotide 638, except that it lacked nucleotides 100 to 165 (Figure
35 1b). In comparing the cDNAs to Factor VII genomic DNA, the absent sequences correspond precisely to one exon-like region. Therefore, two Factor VII cDNAs have been obtained which appear to reflect alternative mRNA splicing events.

The leader encoded by λVII2463 is exceptionally long (60 amino acids) and has a very different hydrophobicity profile when compared with Factor IX, protein C and prothrombin. This leader contains two
40 mets, at positions -60 and -26. Initiation most likely begins at the first met, since a hydrophobic region, typical of signal peptides, follows the met at position -60, but not the met at -26. It is interesting that the absent sequence in λVII565, which corresponds precisely to an exon-like region in the genomic clone, results in a 38 amino acid leader with a hydrophobicity pattern more analogous to Factor IX, protein C, and prothrombin.

45 Since it was not clear then which, if either, of the leaders described above was authentic, an additional approach was initiated in an effort to analyze the 5' end sequence. Briefly, this approach included the construction and screening of a human genomic DNA library, and the identification of genomic clones comprising Factor VII gene sequences. The 5' portion of the genomic sequence was subsequently joined to the cDNA to construct a full-length clone.

50 In an additional construct, a 5' Factor VII cDNA fragment of λVII565 containing all of the leader and 29 amino acids of the mature coding sequence was ligated to a fragment of the cDNA of λVII2463 (containing the remainder of the mature protein and 3'-untranslated sequences). This "565-2463" sequence encodes a full-length Factor VII cDNA sequence as a single EcoRI fragment.

55 The DNA sequences described above are then inserted into a suitable expression vector which is in turn used to transfect a mammalian cell line. Expression vectors for use in carrying out the present invention will comprise a promoter capable of directing the transcription of a foreign gene in a transfected mammalian cell. Viral promoters are preferred due to their efficiency in directing transcription. A particularly preferred such promoter is the major late promoter from adenovirus 2. Such expression vectors will also

contain a set of RNA splice sites located downstream from the promoter and upstream from the insertion site for a gene encoding a protein having biological activity for blood coagulation. Preferred RNA splice site sequences may be obtained from adenovirus and/or immunoglobulin genes. Also contained in the expression vectors is a polyadenylation signal, located downstream of the insertion site. Viral polyadenylation signals are preferred, such as the early or late polyadenylation signals from SV40 or the polyadenylation signal from the adenovirus 5:Elb region. In a particularly preferred embodiment, the expression vector also comprises a viral leader sequence, such as the adenovirus 2 tripartite leader, located between the promoter and the RNA splice sites. Preferred vectors may also include enhancer sequences, such as the SV40 enhancer.

Cloned DNA sequences may then be introduced into cultured mammalian cells by calcium phosphate mediated transfection. (Wigler et al., *Cell* 14: 725, 1978; Corsaro and Pearson, *Somatic Cell Genetics* 7: 603, 1981; Graham and Van der Eb, *Virology* 52: 456, 1973.) A precipitate is formed of the DNA and calcium phosphate and this precipitate is applied to the cells. A portion of the cells take up the DNA and maintain it inside the cell for several days. A small fraction of the cells (typically 10^{-4}) stably integrate the DNA into the genome. In order to identify these stable integrants, a gene that confers a selectable phenotype (a selectable marker) is generally introduced along with the gene of interest. Preferred selectable markers include genes that confer resistance to drugs, such as G-418 and methotrexate. Selectable markers may be introduced into the cell on a separate plasmid at the same time as the gene of interest or they may be introduced on the same plasmid. A preferred selectable marker is the gene for resistance to the drug G-418, which is carried on the plasmid pKO-neo (Southern and Berg, *J. Mol. Appl. Genet.* 1: 327-341, 1982). It may also be advantageous to add additional DNA, known as "carrier DNA," to the mixture which is introduced into the cells. After the cells have taken up the DNA, they are allowed to grow for a period of time, typically 1-2 days, to begin expressing the gene of interest. Drug selection is then applied to select for the growth of cells which are expressing the selectable marker in a stable fashion. Clones of such cells may be screened for expression of the protein of interest.

Factor VII produced by the transfected cells may be removed from the cell culture media by adsorption to barium citrate. Spent medium is mixed with sodium citrate and barium chloride and the precipitate collected. The precipitated material may then be assayed for the presence of the appropriate clotting factor. Further purification may be achieved through immunoabsorption. It is preferred that the immunoabsorption column comprise a high-specificity monoclonal antibody. Alternatively, purification of the barium citrate precipitated material may be accomplished by more conventional biochemical methods or by high-performance liquid chromatography.

Conversion of single-chain Factor VII to active two-chain Factor VIIa may be achieved using Factor XIIa as described by Hedner and Kisiel (*J. Clin. Invest.* 71: 1836-1841, 1983), or with other proteases having trypsin-like specificity (Kisiel and Fujikawa, *Behring Inst. Mitt.* 73: 29-42, 1983).

In summary, the present invention provides a method for the production of proteins having the activity of Factor VIIa using transfected mammalian cells. Gene sequences encoding the specific serine protease domain of Factor VIIa are isolated from cDNA libraries. Sequences encoding the leader peptide and calcium binding domains are isolated from cDNA or genomic libraries or constructed from synthesized oligonucleotides. The sequences are then joined in an appropriate expression vector so as to encode Factor VII. The resulting vector and a plasmid containing a drug resistance marker are co-transfected into appropriate mammalian tissue culture cells. Transfected cells may then be selected by addition of the appropriate drug, such as G-418. The protein products are then purified from the cell growth media and assayed for biological activity in a blood coagulation assay and for immunological cross-reactivity using antibodies prepared against authentic human Factor VII.

To summarize the examples which follow, Example 1 discloses the cloning of a full-length cDNA sequence for Factor VII. Example 2 discloses a partial amino acid sequence of human Factor VII, including the sequence of approximately 30 amino acids at the amino terminus. Example 3 discloses the construction and screening of a human genomic DNA library and the identification of genomic clones comprising Factor VII gene sequences. Example 4 discloses the construction of two hybrid gene segments, each comprising a cDNA fragment encoding the leader peptide of Factor IX and a synthesized double-stranded fragment encoding a consensus calcium binding domain. The hybrid sequences are then joined to partial cDNA clones of Factor VII. Using *in vitro* mutagenesis, the consensus sequence was then altered to conform to the protein sequence data for Factor VII. Example 5 describes the construction of a gene sequence encoding a fusion protein comprising the calcium binding domain of Factor IX and the specific serine protease domain of Factor VII. Example 6 describes the construction of the vector pD2 for use in expressing proteins having biological activity for blood coagulation in transfected mammalian cells. The gene fusion described in Example 5 is expressed using this vector. Example 7 describes the use of the

vector pD2 to express a gene for Factor IX in a transfected mammalian cell line. Example 8 describes the construction of the vector pM7135, which contains DNA sequences encoding a primary translation product comprising the leader sequence of Factor IX fused to Factor VII. This vector may be used to produce a protein having the activity of Factor VII in a transfected mammalian cell line. Example 9 describes the expression of Factor VII using cDNA sequences, and the expression of Factor VII from a genomic-cDNA hybrid sequence.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

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Restriction enzymes were obtained from Bethesda Research Laboratories (BRL) and New England Biolabs and were used as directed by the manufacturer, unless otherwise noted. Oligonucleotides were synthesized on an Applied Biosystems Model 380 A DNA synthesizer and purified by polyacrylamide gel electrophoresis on denaturing gels. *E. coli* cells were transformed as described by Maniatis et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982). M13 and pUC cloning vectors and host strains were obtained from BRL. Factor VII was prepared from human plasma as described by Kisiel and McMullen (*ibid*).

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Example 1: Cloning of a Partial Factor VII cDNA.

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A. Construction of a human liver cDNA library.

A cDNA library was prepared from human liver mRNA by the method of Chandra et al., Proc. Natl. Acad. Sci. U.S.A. 80: 1845-1848, 1983. The cDNA preparation was sedimented through an alkaline sucrose gradient (Monahan et al., Biochemistry 15: 223-233, 1976) and fractions containing species of greater than about 1000 nucleotides were pooled. The first strand preparation was made double-stranded using reverse transcriptase (Chandra et al., 1983), treated with S1 nuclease, and the residual staggered ends filled-in using DNA Polymerase I (Klenow fragment) in the presence of all four deoxyribonucleotide triphosphates (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982). The blunt-ended cDNA was treated with Eco RI methylase and ligated to phosphorylated Eco RI linkers using T₄ DNA ligase (Maniatis et al., *ibid*). The ligated DNA preparation was exhaustively digested with Eco RI to remove excess linker sequences and double-stranded DNAs greater than about 1000 base pairs in length were purified by neutral sucrose gradient centrifugation (Maniatis et al., *ibid*). Native λ gt11 DNA was ligated into concatemers, digested to completion with Eco RI, and the 5' terminal phosphates were removed by treatment with bacterial alkaline phosphatase. The pooled human liver cDNA was ligated with the phage DNA, packaged in vitro (Maniatis et al., *ibid*), and used to infect *E. coli* Y1088 (Young and Davis, Science, 222: 778-782, 1983). Approximately 14×10^6 primary phage plaques were generated in this library, composed of seven libraries of $\sim 2 \times 10^6$ plaques each. Greater than 90% of these were recombinants containing human DNA inserts, based on their lack of β -galactosidase activity and characterization of 20 random clones by Eco RI digestion followed by agarose gel electrophoresis. The cDNA library, in the form of phage particles, was purified by cesium chloride gradient centrifugation and stored in SM buffer (Maniatis et al., *ibid*).

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B. Screening of the human liver cDNA library for Factor VII clones.

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The human liver expression cDNA library described above was screened for specific antigen (Young and Davis, *ibid*) using an ¹²⁵I-labeled monoclonal Factor VII antibody prepared by the method of Brown et al. (J. Biol. Chem. 225: 4980-4983, 1980) using purified Factor VII. Screening of 6×10^6 phage plaques identified one isolate, designated λ VII2115, which gave a positive response with the antibody.

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The phage clone λ VII2115 was tested against two other anti-Factor VII monoclonal antibodies and a rabbit polyclonal antibody to Factor VII. Isolate λ VII2115 gave a positive response to all these anti-Factor VII antibodies.

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DNA was prepared from a plate lysate (Maniatis et al., pp. 65-66, 1982) of λ VII2115. Digestion of this DNA with Eco RI liberated an insert of 2139 base pairs. This insert was subcloned into M13 phage vectors (Messing, Meth. in Enzymology 101: 20-77, 1983; and Norrander et al., Gene 26: 101-106, 1983) for chain termination dideoxy DNA sequencing (Sanger et al., Proc. Natl. Acad. Sci. U.S.A. 74: 5463-5476, 1977). This cDNA insert contains Pst I sites at positions 214, 839, and 1205 (designated Pst Ia, Pst Ib, and Pst Ic, respectively, in Figure 1a) and a Sma I site located at position 611. The following M13 templates were

sequenced:

- 1) full-length (2139 bases) Eco RIa → Eco RIb fragment in M13mp18 (designated clone F7-1);
 - 2) Pst Ia → Eco RIa 214 base fragment in M13mp19 (F7-2);
 - 3) Pst Ia → Pst Ib 625 base fragment in M13mp18 (F7-3);
 - 5 4) Pst Ib → Pst Ia 625 base fragment in M13mp18 (F7-7);
 - 5) Sma I → Pst Ib 228 base fragment in M13mp10 (F7-8);
 - 6) Pst Ib → Pst Ic 366 base fragment in M13mp18 (F7-9);
 - 7) Pst Ic → Pst Ib 366 base fragment in M13mp18 (F7-10);
 - 8) Pst Ic → Eco RIb 930 base fragment in M13mp19 (F7-11); and
 - 10 9) Eco RIb → Eco RIa full-length fragment in M13mp18 (F7-12)
- (restriction site designations refer to Figure 1a).

The data confirmed the sequence on both strands for 91% of the coding region and 15% of the 3' non-coding region and yielded single-stranded sequence information for the remaining 9% of the coding region and 85% of the non-coding region.

- 15 Comparison of the amino acid sequence predicted from the cDNA sequence with the known amino acid sequence data of Kiesel and McMullen (*Thrombosis Research* 22: 375, 1981) and the amino acid sequence shown below (Example 2) revealed an anomaly which could be explained by the absence of three nucleotides in the DNA sequence near position 400. To obtain additional sequence data, λ VII2115 was digested with Eco RI, and the Factor VII coding fragment was inserted into pUC 13 (Vieira and Messing,
- 20 *Gene* 19: 259-268, 1982; and Messing, *ibid*) which had been digested with Eco RI. The resultant recombinant plasmid, designated pUCVII2115, was digested with Xba I which cut at position 328. The digested sample was divided in half: half was labeled with $\alpha^{32}\text{P}$ dCTP and DNA Polymerase I (Klenow fragment) (Englund, P.T., *J. Mol. Bio.* 66: 209, 1972); the other half was labeled with $\gamma^{32}\text{P}$ ATP and polynucleotide kinase (Chaconas et al., *Biochem. Biophys. Res. Comm.* 66: 962, 1975). The labeled
- 25 plasmids were then recut with Pst I to yield 113 and 509 base pair fragments. Both strands of each of these were sequenced by the method of Maxam and Gilbert (*Meth. in Enzymology* 74: 560, 1980). The 113 base pair fragment was sequenced in its entirety and 210 base pairs of the 509 base pair fragment were sequenced. These sequences revealed three additional bases (one C and two G's) which rendered the DNA sequence data in agreement with the protein sequence data, indicating that the previous anomalous results
- 30 arose from compressions on the sequencing gel due to secondary structure involving G's and C's. The sequence of the last 9% of the coding region on both strands was also confirmed.

- Further analysis of the sequence of the pUCVII2115 insert confirmed that a portion of this cloned fragment encoded a sequence of 11 amino acids known to be at the cleavage site of Factor VII (Kiesel and McMullen, *Thrombosis Research* 22: 375, 1981). Comparison of this sequence to Factor IX (Davie et al.,
- 35 *ibid*) and Factor X (Leytus et al., *Proc. Natl. Acad. Sci. U.S.A.* 81: 3699-3702, 1984) amino acid sequences suggested that the clone contained the sequence for Factor VII beginning at (approximately) nucleotides coding for amino acid 36 of the mature Factor VII protein and continuing through approximately 1000 coding and 1100 noncoding nucleotides and poly A sequence. In addition, it was found that this clone had frameshift mutations in the 3' coding portion.

- 40 In order to obtain the correct 3' coding region, all 14 million clones of the seven λ gt11 cDNA libraries were screened by plaque hybridization (Benton and David, *Science* 196: 180-181, 1977) with nick-translated cDNA of λ VII2115 (Maniatis et al., pp. 109-112, 1982).

- Seven positive isolates were then screened by dideoxy sequencing of pUC plasmids into which the cDNA inserts had been subcloned (Wallace et al., *Gene* 16: 21, 1981). The λ gt11 clones were digested with
- 45 Eco RI and the Factor VII fragments were inserted into pUC13 which had been cleaved with Eco RI. All except one of these were found to start at a position corresponding to base 212 of the insert in λ VII2115; the one exception consisted only of 3' non-coding sequence. One of the clones starting at base 212 was selected for analysis and was designated clone pUCVII1923.

- Because analysis of pUCVII2115 indicated the presence of frameshift mutations between positions 657
- 50 and 815, pUCVII1923 was first analyzed in this region by Maxam-Gilbert sequencing. Plasmid pUCVII1923 was digested with Nar I (position 779 in Figure 1a). The cut DNA was labeled with $\alpha^{32}\text{P}$ dCTP using DNA polymerase I (Klenow fragment) and subsequently digested with Ava I (which cleaves at the same site as Sma I in Figure 1) and Taq I (site at 1059), yielding a Nar I-Ava I 166 bp fragment and a 200 bp Nar I-Taq I fragment. Each of these was sequenced. A C, missing in pUCVII2115, was found at position 697 and
- 55 another C, also missing in pUCVII2115, was found at position 798.

The rest of the sequence of the coding region of pUCVII1923 was shown to be correct by sequencing by the dideoxy method on an M13 subclone of the entire insert of pUCVII1923. The Lac primer ZC87 (Table 1) was used to sequence from position 212 (Figure 1a) to 512; primer ZC218 (CTCTGCCTGCCGAAC) was

used to sequence from 715 to 1140 and primer ZC217 (ATGAGAAGCGCACGAAG) was used for sequencing from 720 to 350. Since the pUCVII2115 insert is correct from position 13 (positions 1-12 include an artificial linker) to 695, and pUCVII1923 is correct from position 212 to the end, the two were spliced together to yield a molecule correct from position 13 (Figure 1a) to the end. A convenient point utilized for this splice is the Xba I site at position 328. The sequence of the spliced corrected molecule is shown in Figure 1a.

Because a full-length Factor VII clone was difficult to obtain by cDNA cloning, three strategies were adopted to provide the missing coding sequence and the necessary upstream processing and signal sequences. The first strategy was to obtain the needed sequence from a human genomic DNA library or through additional screening of cDNA libraries. The second approach was to synthesize the necessary 5' coding sequence, based on the amino acid sequence data for Factor VII (Example 2) and the published sequences of the genes encoding vitamin K-dependent clotting factors (Kurachi and Davie, *ibid*; and Davie et al., *ibid*), and join this to a portion of the prepro sequence of Factor IX. The third strategy relies on the functional homology of the amino terminal regions of Factor VII and Factor IX. A sequence was constructed which comprised the coding regions for the leader and amino-terminal portion of Factor IX. This was then fused in the proper orientation to the partial Factor VII cDNA.

In order to obtain DNA sequences that comprise the entire DNA sequence of Factor VII, an attempt was made to isolate the remaining 5' DNA sequence. This was accomplished through the utilization of the 5' terminal 0.3 kb EcoRI-XbaI fragment from the cDNA insert of λ VII2115 to screen a cDNA library comprising 2×10^6 phage. The library was constructed using poly(A) mRNA from HepG2 cells following an adaptation of the method of Gubler and Hoffman (Gene 25: 263-269, 1983). The RNA was reverse transcribed to generate first strand cDNA, followed by second strand synthesis using DNA polymerase I and RNase H. Following EcoRI methylation and passage over a Sepharose® 6B column, the DNA termini were blunted with T₄ DNA polymerase. EcoRI linkers were added and excess linkers were removed by digestion with EcoRI and chromatography on Sepharose® CL 2B. The DNA in the void volume was collected and ligated to λ gt11 which had been digested with EcoRI and treated with calf intestinal phosphatase. The DNA was packaged and infected into E. coli Y1088. Several positives were detected, and the EcoRI fragments were subsequently subcloned into M13 phage vectors for dideoxy sequencing using either the M13 universal primer or Factor VII specific oligonucleotides.

From these, three new cDNA clones of Factor VII were obtained, and their sequences completely determined. The largest of these cDNAs, from a clone designated λ VII2463, was found to contain the entire coding sequence for Factor VII. This clone included a 35 nucleotide 5' untranslated region, 180 nucleotides coding for a 60 amino acid leader, 1218 nucleotides coding for the 406 amino acid mature protein, a stop codon, 1026 nucleotides of 3' untranslated sequence, and a 20 base poly(A) tail (beginning at position 2463). This cDNA has now been sequenced in its entirety on both strands. A comparison of it with two cDNAs isolated earlier from clones λ VII2115 and λ VII1923 revealed that clone λ VII2463 contains an additional 321 nucleotides upstream of the insert in λ VII2115 and 519 nucleotides upstream of the insert in λ VII1923. The overlapping Factor VII sequences of λ VII2463 and these two previous cDNAs agree, except that the cDNA of λ VII2463 does not contain single base deletions at positions 1005 and 1106, which were detected in the cDNA of λ VII2115. Thus, λ VII2463 contains, on a single EcoRI fragment, a Factor VII cDNA coding for Factor VII leader and mature protein sequences.

An additional cDNA, λ VII565, was isolated and found to contain 5' terminal Factor VII sequences, but was truncated within the coding sequences. Its 5' end maps at nucleotide 9 (Figure 1b).

When compared with full-length λ VII2463, λ VII565 was found to lack a sequence corresponding to one exon-like region within the leader sequence. Bases 100-165 are absent from λ VII565 (Figure 1b). The absent sequences correspond precisely to one exon-like region by comparison with genomic sequence data (as described in Example III). Thus, the λ VII565 structure may be a consequence of alternative splicing events in the leader sequence.

The leader encoded by λ VII2463 is exceptionally long (60 amino acids) and has a very different hydrophobicity profile when compared with Factor IX, protein C and prothrombin. This leader contains two Mets, at positions -60 and -26. Initiation most likely begins at the first Met, since a hydrophobic region, typical of signal peptides, follows the Met at position -60, but not the Met at -26. It is interesting that the absent sequences in λ VII565, which corresponds precisely to an exon-like region in the genomic clone, results in a 38 amino acid leader with a hydrophobicity pattern more analogous to the above proteins.

Example 2: Amino Acid Sequence of Human Factor VII.

The elucidation of the amino acid sequence of human Factor VII was desired in order to confirm the identity of putative cDNA clones, substantiate the sequence of Factor VII cDNA, provide information allowing for the synthesis of specific oligonucleotide probes to screen cDNA and genomic libraries for clones containing the 5' sequence, and to construct a synthetic fragment encoding the amino-terminal portion of Factor VII. Although limited amino acid sequence was provided by Kisiel and McMullen (ibid), more information was needed.

Purified human Factor VIIa (Kisiel and McMullen, ibid) was reduced and carboxymethylated by the method of Crestfield et al., *J. Biol. Chem.* 238: 622, 1963. The light and heavy polypeptide chains of carboxymethylated Factor VIIa were separated by high-performance liquid chromatography (HPLC) on a Micro Pak C18 reverse phase column (Varian Corp.) by generating a gradient of 0.1% TFA in distilled water (A) and 0.1% TFA in acetonitrile (B) from 0-40% B in 5 minutes, 40-80% B in 25 minutes and 80-100% B in 5 minutes. Approximately 300 picomoles of each peptide chain were analyzed by automated Edman degradation using a Gas-Phase Protein Sequencer (Applied Biosystems, Inc.). Eighteen and 29 residues were identified at the amino-termini of the heavy and light polypeptide chains, respectively. The amino-terminal sequence of the heavy chain of Factor VIIa was consistent with that encoded by cDNA clone pUCVII2115 (Figure 2b). Amino acid residues are designated within Figures 2a and 2b by single letter code as follows: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine; X indicates an unknown residue and * indicates that the Glu residues (γ) were assigned by homology to the structures of other known clotting factors and by the absence of any other phenylthiohydantoin-amino acid at those positions. The gaps (-) are placed to provide the best alignment among the sequences. In addition, the information indicated that the amino acids at positions five and nine were lysines and not threonine and arginine, respectively, as previously reported (Kisiel and McMullen, ibid). The sequence analyses of the light chain of Factor VIIa, which originates from the amino-terminal region of Factor VII, fell short by approximately 6 residues to overlap with the structure encoded by the 5' end of cDNA clone pUCVII2115.

To obtain additional sequence data, two nanomoles of the carboxymethylated light chain were digested for 12 hours by bovine chymotrypsin (1:100 w/w, enzyme: substrate) in 0.1 M ammonium bicarbonate, pH 7.8, at 37°C. The generated fragments were purified by HPLC on a Micro Pak C18 reverse phase column using the above solvents in a gradient of 0-30% B in 5 minutes, 30-60% B in 25 minutes and 60-80% B in 10 minutes. Peptides were identified by their U.V. absorption at 220 and 280 nm. Lyophilized peptides (approximately 1 nanomole each) were analyzed by Edman degradation. The results (Figure 2b) confirmed much of the cDNA sequence in the corresponding region of clone pUCVII2115. In total, 113 of 152 residues (75%) of the light peptide chain of Factor VIIa were identified. This sequence is identical to that encoded by the known cDNA structure. Indirect evidence indicates Asn 145 is a site of carbohydrate attachment.

Example 3: Cloning of the genomic Factor VII sequence.

As one approach to providing the 5' end sequence lacking from the cDNA, a lambda phage library containing human fetal liver DNA (Lawn et al., *Cell* 15: 1157-1174) was screened with nick translated Factor VII cDNA. A portion of the genomic library was plated on *E. coli* LE392 (ATCC 33572) to produce a total of 7.2×10^6 plaques (Maniatis et al., ibid, pp. 320-321). The phage plaques were adsorbed from the plates onto nitrocellulose and hybridized with the ^{32}P -labeled cDNA according to the procedure of Benton and Davis (*Science* 196: 180, 1977). Eight clones were obtained and plaque purified.

Using a DNA fragment (Eco RI-Xba I, Figure 1) from the 5' end of the Factor VII cDNA (λ VII2115) and standard techniques (Maniatis et al., ibid) those genomic clones containing 5' end sequences were identified. These phage were designated 7m1, 7m2 and 7m3. DNA was prepared from these recombinant phage and preliminary restriction endonuclease maps derived. Phage 7m1, which gave the strongest hybridization signal, was used to generate a more extensive restriction map and to place the Eco RI-Xba I cDNA sequences on this map by Southern blotting (Southern, *J. Mol. Biol.* 98: 503, 1975).

In order to determine if phage 7m1 contained the DNA sequences encoding the amino terminal amino acids of the Factor VII protein, Southern blots of phage DNA restriction digests were hybridized with mixtures of oligonucleotides whose sequences were deduced from the Factor VII amino terminal amino acid sequence. Oligonucleotides ZC188, ZC360, and ZC401 (Table 1) were radioactively labeled with T_4 polynucleotide kinase and hybridized to the phage DNA blots at a few degrees centigrade below their T_m (Wallace, R. B., et al., *Nuc. Acids Res.* 6: 3543-3557, 1979). The results of this analysis indicated that a 3.7

kb Sst I fragment of 7m1 contained sequences hybridizing to these oligonucleotides. This Sst I fragment was subcloned into M13 for DNA sequence analysis. Results obtained using ZC360 as sequencing primer identified a region approximately 60 nucleotides in length, which corresponded to the amino-terminal protein sequence data.

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	<u>Oligonucleotide</u>	<u>Sequence</u>
10	ZC87	TCC CAG TCA CGA CGT
	ZC188	GCC GGG ^T CTCA ^G CTC CTC CA ^A GAA GGC GTTGG C A G
15	ZC212	GAC CTG CAG GAT CCA TGC AGC GCG TGA ACA TGA TCA TGG
20	ZC213	GAG GCC TGG TGA TTC TGC CAT GAT CAT GTT CAC GCG CTG
	ZC217	ATG AGA AGC GCA CGA AG
25	ZC218	CTC TGC CTG CCG AAC
	ZC235	GAT CCA TGC AGC GC
	ZC249	AGA ACA GCT TTG TTC TTT CA
30	ZC275	GCC CCC ATT CTG GCA
	ZC286	CCA AAG AGG GCC AAC GCC TTC CTG GAG GAG AGA CCT GGG AGC CTG GAG AGA GAG TGT ATT GAG G
35	ZC287	AAT ACA CTC TCT CTC CAG GCT CCC AGG TCT CTC CTC CAG GAA GGC GTT GGC CCT CTT TGG
	ZC288	AGC AGT GTA GCT TCG AGG AGA ACA GAG AGG TTT TCG AGG CCA GCG ACG
40	ZC289	AAT TCG TCG CTG GCC TCG AAA ACC TCT CTG TTC TCC TCG AAG CTA CAC TGC TCC
45	ZC333	CAG CTT CGT CCT GTC GCT GGC CTC

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ZC336 CCT CTT TGG GCC TGG TGA

5 ZC360 $\begin{matrix} C & C & C & C & G \\ CA & TC & TC & TC & TT & CA \\ T & T & T & T & A \end{matrix}$

10 ZC401 CGT AGC GTT CAG GCC CTC GAA GAT CTC GCG GGC
CTC CTC GAA GCT ACA C

15 Since genomic clone 7m1 was known to contain 7kb of sequences upstream of exon 2, this clone was anticipated to encode Factor VII 5'-untranslated sequences and the leader sequences up to the amino acid position -17. In order to confirm that exon 1 was encoded within genomic clone 7 m1, the leader sequence information from clones λ VII2463 and λ VII565 was used to design oligonucleotides ZC528 and ZC529 (shown below).

20 ZC528
5'TCA ACA GGC AGG GGC AGC ACT GCA GAG ATT^{3'}

25 ZC529
5' TTC CAC GGC ATG TCC CGT GTT TCT CCT CCT^{3'}

30 These were used to probe 7m1 DNA, and a subclone, 7SD, was found that hybridized to both oligonucleotides. Exon 1 was determined to be composed of two exonic sequences: exon 1a, which hybridized to ZC528 (corresponding to nucleotides 1 to 30 in λ VII2463), and exon 1b, which hybridized to ZC529 (corresponding to nucleotides 119 to 148 in λ VII2463). The intron sequences flanking both exons 1a and 1b have been sequenced: 1a contains a consensus splice donor sequence at the 3' end of the exon, and 1b is flanked on each terminus with a consensus splice acceptor (upstream of 1b) or donor (downstream of 1b) sequence. The position of exon 1a within genomic clone 7m1 has been precisely mapped, while that of exon 1b has been mapped within a defined region. Exon 1b sequences are present in λ VII2463, while λ VII565 appears to be derived from RNA spliced between exon 1a and exon 2, looping out the 1b exonic sequence.

40 A variety of 7m1 subclones in pUC and M13 vectors were prepared to facilitate sequencing the remaining exons. Appropriate oligonucleotides designed from the cDNA sequences, which correspond to exons 1 through 7, were used to sequence all but the last exon. The genomic sequence corresponds exactly to the cDNA sequences through these regions. In addition, the intron/exon boundaries for exons 1-7 have been determined, and most are now precisely mapped within clone 7m1. The intron sizes and positions within the Factor VII gene are listed in Table 2.

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Intron/Exon Junctions in the Factor VII Gene

	<u>Intron</u>	<u>Amino Acid Position</u>	<u>Intron Size (Kb)</u>
5	A	-39	>0.2
	B	-17	>1.0
10	C	37/38	1.92
	D	46	0.068
	E	84	~2
15	F	131	~1
	G	167/168	0.56
	H	209	1.31

20 Phage 7m1 was known to lack the Factor VII 3'-terminus, which includes exon 8. In order to obtain these sequences, a 12-13 kb-enriched Bam HI library in λ L47.1 (Loenen and Brammer, *Gene* 10: 249, 1980; Maniatis, et al., *ibid.*), derived from human dermal primary fibroblast cells, was probed with two nick-translated Factor VII cDNA PstI fragments (corresponding to sequences in exon 7, and to 3'-untranslated sequences). A clone, designated 7DC1, was detected by both probes. Subsequent restriction endonuclease and Southern blot analysis established that clone 7DC1 overlaps with, and extends approximately 3 Kb beyond the terminus of, clone 7m1, and that it contains exon 8. The 3.9 Kb (XbaI-BamHI) fragment from 7DC1 DNA containing exon 8 was subcloned into M13, and sequence analysis was performed using oligonucleotides complementary to its 5' and 3' termini. The entire exon sequence is present in this clone.

30 Example 4: Factor IX-Factor VII Hybrid Genes Containing a Synthesized Coding Sequence.

A. Construction of a hybrid Factor IX leader-synthetic Factor VII 5' coding sequence.

35 The second alternative for obtaining the 5' coding sequence for Factor VII was synthesis of an appropriate double-stranded fragment, using a nucleotide sequence predicted on the basis of the amino terminal amino acid sequence of Factor VII, the amino acid sequences of other vitamin K-dependent clotting factors, and the known nucleotide sequences of other vitamin K-dependent clotting factor genes (Kurachi and Davie, *ibid.*; Anson et al., *EMBO J.* 3: 1053-1060, 1984; and Davie et al., *ibid.*). In order to provide the necessary secretion and processing signals for secretion of a mature Factor VII analog, this synthetic fragment (the consensus sequence) was joined to one of two leader sequences derived from a Factor IX cDNA clone. This strategy is outlined in Figure 3.

40 A cDNA coding for human Factor IX was obtained from a library made with mRNA from human liver (Kurachi and Davie, *ibid.*). The Factor IX sequence was isolated from the pBR322 vector by digestion with Pst I and was inserted into the Pst I site of pUC13. This plasmid was designated FIX-pUC13. In order to remove the G-rich region which was present at the 5' end of the Factor IX insert as a result of cDNA cloning, a synthetic oligonucleotide adaptor was substituted for the 5' end of the cloned fragment. Oligonucleotides ZC212 and ZC213 (Table 1) were synthesized and annealed to generate a 22 base pair overlap, the fragment ends filled in and cut with appropriate restriction endonucleases, and the resulting fragment was joined to the Factor IX sequence.

50 To construct the adaptor, 100 pmoles each of ZC212 and ZC213 were lyophilized and resuspended in 10 μ l of 10x kinase/ligase buffer (600 mM Tris pH 8.0, 100 mM MgCl₂, 100 mM DTT) plus 86 μ l H₂O. The annealing reaction was run at 65°C for 10 minutes, the mixture was slowly cooled to room temperature and put on ice. To this mixture was added 4 μ l of 2.5 mM dNTP mix and 1 μ l (8 units) T₄ DNA polymerase. The reaction was allowed to proceed 45 minutes at 14°C. Ten μ l of 5 M NH₄OAc was then added and the DNA was extracted once with phenol/CHCl₃, twice with CHCl₃, and was precipitated with ethanol. The DNA was centrifuged and resuspended in 100 μ l medium salt buffer (Maniatis et al., *ibid.*, p. 100), digested with 9 units Pst I and 8 units Cfo I, and extracted as above.

The modified Factor IX sequence was then constructed by combining 0.16 pmoles of the synthetic Pst I-Cfo I adaptor fragment, 0.14 pmoles of a 1.4 kb Cfo I-Bam HI Factor IX fragment from FIX-pUC13, and 0.14 pmoles of a 2.7 kb Bam HI-Pst I pUC13 vector fragment in a 20 μ l reaction containing 60 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 10 mM DTT, and 0.9 units T₄ ligase. The reaction was incubated for 3 h at room temperature and used to transform competent *E. coli* JM83 (Messing, Recombinant DNA Technical Bulletin, NIH Publication No. 79-99, 2, No. 2, 43-48, 1979). The cells were plated with 50 μ l of 2% X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) on L-broth containing 40 μ g/ml ampicillin and incubated at 37°C overnight. White colonies were picked onto another plate containing ampicillin and grown at 37°C overnight. The colonies were blotted on Whatman 540 paper and the paper prepared for hybridization according to the method of Wallace et al. (Gene 16: 21, 1981), except the overnight incubation on chloramphenicol plates was omitted. The papers were incubated at 44°C for 2 h in 0.9 M NaCl, 0.09 M Tris-HCl pH 7.5, 6 mM EDTA, 0.5% Nonidet P-40, 150 μ g/ml *E. coli* tRNA. The papers were probed with ³²P-labeled ZC235 (Table 1), a 14-mer that is specific for the altered 5' end sequence. Hybridization with 1-2 x 10⁶ cpm per filter was carried out at 44°C in the prehybridization buffer overnight. The filters were then washed 3 times in 6 x SSC, 0.1% SDS at 4°C and 3 times in 2 x SSC, 0.1% SDS at 44°C and exposed to X-ray film. Two positive clones were obtained. One of these clones was designated FIX (-G) \rightarrow pUC13.

In order to confirm the sequence of the altered region of the Factor IX portion of the FIX(-G) \rightarrow pUC13 construct, dideoxy sequencing directly on the pUC plasmid using the BRL reverse primer was performed using the method of Wallace et al., 1981 (ibid) using a primer end labeled with polynucleotide kinase and ³²P ATP by the method of Chaconas et al. (ibid). The sequence was as predicted.

The resulting recombinant plasmid contains three Hae III cleavage sites, the first at position 39 in the Factor IX sequence (numbering is based on the published sequence of Anson et al. (ibid), beginning at the first ATG), the second at position 130, and a third in the pUC13 polylinker. The site at 130 is a single base pair upstream from the codons for the Lys-Arg processing site of the prepro Factor IX molecule. In the final Factor IX-Factor VII hybrid constructs, the Factor IX leader sequence, terminated at position 39 or 130, was joined to a synthetic double-stranded fragment comprising the predicted consensus sequence and the last 3 codons of the Factor IX leader sequence.

The synthetic consensus fragment was produced by joining oligonucleotides ZC286-ZC289 (Table 1) to form a double-stranded fragment. One hundred pmole of each oligonucleotide was lyophilized and resuspended in 20 μ l of 1x kinase buffer and incubated overnight at 4°C; then heated at 65°C for 10 minutes. Two pools were made using the kinased oligonucleotides. Pool 1 contained ZC286 + ZC287; pool 2 contained ZC288 + ZC289. The pooled pairs were annealed 10 minutes at 65°C, then cooled to room temperature over a period of 2 hours and placed on ice for 30 minutes.

The modified Factor IX fragment was removed from FIX(-G) \rightarrow pUC13 as a Hind III-Eco RI fragment. Approximately 20 μ g of plasmid was digested with 30 units each of Hind III and Eco RI in 100 μ l Hind III buffer (BRL) containing 4 μ g RNase A at 37°C overnight. The reaction was terminated by heating at 65°C for 10 minutes, and the vector and Factor IX fragments were electrophoresed on a 1% agarose gel and purified by electro-elution. The Factor IX fragment was precipitated with ethanol, resuspended in buffer containing 400 ng/ μ l RNase A, and digested with 9 units of Hae III overnight at 37°C. The Hind III-Hae III 39 base pair Factor IX fragment was isolated from this digest by electrophoresis on a 1.5% agarose gel followed by electro-elution. To obtain the Hind III-Hae III 130 base pair Factor IX fragment, FIX-pUC13 was digested with Eco RI and Hind III and the Factor IX fragment isolated as above. Approximately 3 μ g of this Hind III-Eco RI fragment was digested with 6 units of Hae III at 37°C and aliquots were removed at five minute intervals over 30 minutes into a solution containing 50 mM EDTA. The aliquots were pooled and the Hind III-Hae III 130 base pair fragment was purified by electrophoresis on a 5% acrylamide gel followed by electro-elution.

The final Factor IX-consensus sequence hybrids were prepared by joining, in a four-part ligation, oligonucleotide pools 1 and 2, Factor IX Hind III-Hae III (39 or 130 base pairs), and pUC13 Hind III-Eco RI. The resulting plasmids were used to transform *E. coli* HB101 (ATCC 33694). Colonies were screened by digestion of DNA with Eco RI and Hind III. The sequence comprising the 39 base pair Factor IX sequence joined to the synthetic consensus sequence is hereinafter referred to as mini-FIX-FVII. The plasmid containing this construct was designated pM7200(-C). The sequence comprising the 130 base pair Factor IX sequence joined to the synthetic consensus sequence is referred to as maxi-FIX-FVII. The plasmid containing this construct was designated pM7100(-C). The consensus sequence encodes a polypeptide comprising the amino acid sequence Ala-Asn-Ala-Phe-Leu-Gla-Gla-Arg-Pro-Gly-Ser-Leu-Gla-Arg-Gla-Cys-Lys-Gla-Gln-Cys-Ser-Phe-Gla-Gla-Ala-Arg-Gla-Ile-Phe-Gla-Gly-Leu-Asn-Arg-Thr-Lys-Leu.

B. Joining Factor IX-consensus sequence hybrid fragment to Factor VII cDNA clone.

The Factor IX-consensus sequence hybrids (either mini or maxi) were joined to the 5' portion of the Factor VII cDNA and the vector pUC13 in a three-part ligation (Figures 4 and 5). The vector fragment was produced by digesting 6 ug of pUC13 with 10 units each of Xba I and Hind III in Hind III buffer containing RNase A (400 ng/ul). The mini-FIX-FVII fragment was produced by digesting 2 ug of pM7200(-C) with 10 units each of Hind III and Eco RI as above. The maxi-FIX- FVII fragment was similarly prepared from pM7100(-C). The 5' portion of the Factor VII cDNA was prepared from a plasmid (pUCG705) comprising the Eco RI-Xba I 5' fragment of pUCVII2115 subcloned into pUC13 by digestion with Xba I and Eco RI. Digests were run at 37 °C for 2 hours and the products were separated by electrophoresis on a 1.5% agarose gel. The desired fragments were electro- eluted, extracted with phenol/CHCl₃ and CHCl₃, and precipitated with ethanol. The three fragments, pUC13/Xba I-Hind III, Factor IX-Factor VII (mini or maxi)/Hind III-Eco RI, and 5' Factor VII/Eco RI-Xba I were then ligated in 20 ul of ligase buffer containing 2 ul 20 mM ATP and 0.9 unit T₄ DNA ligase overnight at 4 °C. Colonies were screened by restriction analysis with Hind III and Xba I. The recombinant plasmids containing the mini- and maxi-FIX-FVII sequences were designated pM7200 and pM7100, respectively (Figure 4).

Due to the linker addition used in producing the Factor VII cDNA, modifications had to be made in the fusion sequences to generate correct in frame coding sequences. Both mini- and maxi-fusions contain an Eco RI site at the junction between the Factor IX-consensus sequence hybrid and the Factor VII cDNA which is an artifact of the cDNA cloning process. In addition, the mini-fusion requires the addition of a C to change the sequence at the Hae III site from 5'AGGCCA3' to 5'AGGCCCA3' and establish the correct reading frame downstream of this sequence. These corrections were made by oligonucleotide-directed site specific mutagenesis, essentially as described for the two-primer method by Zoller and Smith (Manual for Advanced Techniques in Molecular Cloning Course, Cold Spring Harbor Laboratory, 1983). The mini-FIX-FVII fragment was removed from pM7200 by digestion with Hind III and Xba I and inserted into M13mp19. The maxi-FIX-FVII fragment was purified from pM7100 and subcloned in a similar manner. The mutagenic primers ZC333 and ZC336 (see Table I) were used for removal of the Eco RI site and the base insertion, respectively. In each case, the universal primer ZC87 was used as the second primer. The mutagenic primers were phosphorylated by combining 40 pmoles of primer and 60 pmoles ATP with 1 unit of T₄ DNA kinase overnight at 60 °C. To remove the Eco RI site from the maxi-FIX-FVII hybrid, 1 ug of the M13 single-stranded template was combined with 20 pmoles each ZC333 and ZC87 in a total volume of 10 ul. The primers were annealed to the template for 10 minutes at 65 °C, cooled to room temperature for 5 minutes, then placed on ice for 5 minutes. The primers were extended using DNA polymerase I (Klenow fragment). To remove the Eco RI site and correct the reading frame in the mini-FIX-FVII hybrid, 1 ug of the appropriate M13 single-stranded template was combined with 20 pmoles each ZC333, ZC336 and ZC87. Annealing and primer extension reactions were carried out as described above. Plaque lifts were screened with ³²P-labeled primer (ZC333 or ZC336) at 60 °C and sequences confirmed by dideoxy sequencing. The resultant constructs, comprising the maxi- and mini-FIX-FVII sequences, were designated pM7111 and pM7211, respectively.

The consensus sequence contains several regions which do not conform to the protein sequence data obtained for Factor VII (Figure 2). In order to produce a sequence which encodes a polypeptide with greater homology to the amino-terminal portion of Factor VII, the consensus sequence was altered by oligonucleotide-directed site-specific mutagenesis. The changes made were the insertion of Leu at position 8, substitution of Ile for Lys at position 18 (numbers refer to the amino acid position after the insertion at position 8), Asn for Ala at position 26, and the sequence Ala-Ser-Asp for Gly-Leu-Asn at positions 32-34 (based on tentative amino acid sequence data).

The sequence changes at positions 8 and 18 were made using pM7111 (sense strand) as template. Primers ZC352 (5' CCC AGG TCT CAG CTC CTC CAG3') and ZC353 (5' CTG CTC CTC CTT ACA CTC TCT3') were annealed to the template and extended as described above. The resultant phage clone was designated pM7114. The sequence of the insert in pM7114 was confirmed by dideoxy sequencing.

In a similar manner, the changes at positions 26-34 were made on the pM7114 template (sense strand) using the mutagenic primer ZC366 (5'CAG CTT CGT CCT GTT CAG GCC CTC GAA GAT CTC GCG GGC CTC CTC GAA3') and ZC87 (Table 1) as second primer. The resultant construct was designated pM7115. The sequence of the entire 550 bp insert in the M13 vector was determined by dideoxy sequencing and found to be correct.

Example 5: Construction of Factor IX-Factor VII cDNA fusion.

The Factor IX-Factor VII cDNA fusion was prepared using Factor IX cDNA obtained from a human liver cDNA library as described by Kurachi and Davie (*ibid*) and the Factor VII cDNA sequence described in Example 1.

The fusion point chosen for the hybrid protein was between amino acid +38 (threonine) of Factor IX and the first lysine encoded by the Factor VII cDNA sequence. Such a protein would be encoded by a sequence consisting of the first 252 bp of the Factor IX cDNA sequence and all of the pUCVII2115 Factor VII cDNA sequence except the first two codons. To construct this hybrid sequence, the Factor IX sequence was first fused to pUCVII2115 using convenient restriction sites. This fusion resulted in the plasmid FIX/VII/12 (described below) which contains the first 310 bp of the Factor IX cDNA joined to the entire Factor VII cDNA sequence. To achieve the precise junction desired for the hybrid protein, the intervening base pairs were removed by oligonucleotide-directed mutagenesis.

Joining of the Factor IX cDNA sequence to the Factor VII cDNA sequence was accomplished by ligating a 0.3 kb Hind III-Aha III fragment of FIX (-G) → pUC13 (Example 4) to a 4.7 kb Sma I-Hind III fragment from pUCVII2115 (Figure 5). The Hind III-Aha III fragment was prepared by digesting 3 ug of FIX(-G) → pUC13 with 40 units of Hind III in 40 ul of medium salt buffer (Maniatis et al., *ibid*) at 37 °C, 4 hours. The volume was then increased to 100 ul of medium salt buffer, and 5 units of Aha III were added and the 37 °C incubation continued for 18 hours. The DNA fragments were separated by electrophoresis in 1% agarose and the 0.3 kb band isolated as described above. A Sma I partial digestion of pUCVII2115 was obtained by incubating 3 ug of pUCVII2115 at 25 °C for 1 hour with 4.8 units of Sma I in a reaction volume of 30 ul. The reaction was stopped by a 15-minute incubation at 65 °C. The sample was then extracted once with an equal volume of phenol and ethanol precipitated.

The precipitate was collected by a 10-minute microfuge spin, rinsed with 70% ethanol and air dried. The DNA was redissolved in 30 ul of medium salt buffer and digested with 30 units of Hind III at 37 °C for 3 hours. The DNA was subjected to electrophoresis in 0.7% agarose and the 4.7 kb Hind III-Sma I fragment isolated as described above. Equimolar amounts of the two fragments (0.048 pmoles) were ligated in a 10 ul reaction containing 50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 1 mM DTT, 1 mM ATP, and 3 units of T₄ DNA ligase at 14 °C for 3.5 hours and then used to transform competent *E. coli* RRI (ATCC 31343). The cells were grown on ampicillin plates and 12 of the resulting colonies were screened by restriction enzyme digestion for the presence of the desired plasmid construction. DNA from colony 12 (FIX/VII/12) gave the expected restriction enzyme digestion pattern and was used in the next step of the hybrid gene construction.

The oligonucleotide-directed mutagenesis procedure was performed on a single-stranded DNA template. Thus, it was necessary to clone the fused Factor IX/Factor VII sequences into M13mp19. To obtain a conveniently small DNA fragment, a 640 bp Hind III-Xba I fragment was isolated from FIX/VII/12. This fragment contains 310 bp of the 5' end of Factor IX cDNA and 330 bp of the Factor VII sequence. The vector was prepared by digesting 1 ug of M13mp19 RF DNA with 20 units of Hind III and 20 units of Xba I in 40 ul of medium salt buffer at 37 °C for 18 hours. The DNA was subjected to electrophoresis in 1.2% agarose and the linear 6.4 kb fragment isolated from the gel as described above. Five ug of FIX/VII/12 DNA was digested with 10 units of Xba I in 40 ul of medium salt buffer at 37 °C for 18 hours. Twenty units of Hind III were added and the digestion continued at 37 °C for an additional 7 hours. The resulting fragments were separated by electrophoresis in 1.2% agarose and the 640 bp fragment eluted as above. Ten ng of linearized m13mp19 and 1 ng of the 640 bp fragment were ligated at 14 °C for 1 hour and then used to transform competent *E. coli* JM101 (Messing, *Meth. in Enzymology*, *ibid*). The cells were plated with X-gal and IPTG (Messing, *Meth. in Enzymology*, *ibid*) and eight light blue plaques were picked and used to infect 2.5 ml cultures of *E. coli* JM103 at A₆₀₀ = 0.3. After 18 hours' growth at 37 °C, the cells were harvested by centrifugation in a room temperature clinical centrifuge and 20 ul of the supernatant which contains the M13 phage was mixed with 10 ug/l ethidium bromide. By comparison with known standards, each of the eight clones had an insert of approximately the correct size. Single-stranded DNA was then prepared from 1.5 ml of the supernatants as described by Messing (*Meth. in Enzymology*, *ibid*). This construct was then sequenced by the dideoxy method using the oligonucleotide ZC87 as a primer to confirm that the insert junction was correct. One of the correct clones (#4) was used as a template in oligonucleotide-directed mutagenesis to produce a functional Factor IX-Factor VII fusion.

The oligonucleotide ZC249, a 20-mer consisting of 10 bp of the desired Factor IX sequence and 10 bp of the desired Factor VII sequence (Table I) was used as the mutagenic primer. The oligonucleotide ZC87, which hybridizes to the M13mp19 sequence, was used as the second primer.

The mutagenesis procedure was modified from that of Zoller and Smith (ibid). For the annealing reaction, 20 pmoles of ZC249 were phosphorylated by incubating overnight at 4°C in 20 µl 60 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 1 mM DTT, 1mM ATP, 1 unit T₄ kinase. The reaction was stopped by incubation at 65°C for 15 minutes, and the sample was lyophilized. One pmole of single-stranded clone #4 template and 20 pmole of ZC87 were added in 10 µl annealing buffer (200 mM Tris-HCl pH 7.5, 100 mM MgCl₂, 500 mM NaCl, 10 mM DTT). The sample was heated to 65°C for 10 minutes, incubated at room temperature for 5 minutes, and then placed on ice. Ten µl of the following solution was prepared fresh and added to the sample: 20 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 10 mM DTT, 1 mM dNTPs, 1 mM ATP, 0.15 units/µl T₄ DNA ligase, 0.25 units/µl *E. coli* DNA Polymerase I (Klenow fragment). The reaction was then incubated at 15°C for 3 hours and the sample used to transform competent *E. coli* JM101 (Messing, *Meth. in-Enzymology*, ibid).

The resulting plaques were lifted onto nitrocellulose and screened by hybridization to ³²P-labeled ZC249. Dry BA85 filters (Schleicher & Schuell, 0.45 µm) were laid onto the agar plate and the phage allowed to adsorb for 5 minutes. The filters were removed and allowed to dry for 5 minutes, placed on Whatman 3 MM paper, saturated in 0.5 M NaOH, 1.5 M NaCl for 5 minutes, air dried for 3 minutes, placed on Whatman paper, saturated in 1 M Tris-HCl pH 8, 1.5 M NaCl, for 5 minutes, and air dried for 3 minutes. The Tris-HCl step was repeated and the filters were rinsed in 100 ml 6 x SSC for 2 minutes at room temperature. After air drying, the filters were baked at 80°C for 2 hours and prehybridized at 47°C (T_m-4°C of ZC249) overnight in 6.7 x SSC pH 6.5, 2 mg/ml *E. coli* tRNA, and 0.2% (w/v) each BSA, Ficoll, and polyvinylpyrrolidone.

After the prehybridization step, the filters were incubated with 2.5 x 10⁶ cpm/filter of labeled ZC249 in the same SSC hybridization buffer at 47°C overnight. Following hybridization, the filters were washed 3 times, 5-10 minutes each, at room temperature in 6 x SSC and exposed to X-ray film. Putative positive plaques were replated and screened as above. Individual plaques were then picked, and single-stranded DNA was prepared and sequenced using ZC275 as a primer. The oligonucleotide ZC275 corresponds to a sequence 40 bp in the 5' direction of ZC249 on the same strand (Table 1).

Four positive plaques were identified. The entire insert in M13mp19 for one clone (FIX/VII-9) was sequenced by the dideoxy method using the oligonucleotides ZC87 and ZC275 and determined to be correct. The confirmed sequence is represented by bases 1-567 in Figure 7. RF DNA from this clone was then used for the final step in the construction of the hybrid gene.

Three fragments were used to make the final construction: the 0.6 kb Hind III-Xba I fragment from FIX/VII-9 containing the fused IX/VII sequences; a 1.7 kb Xba I-Bam HI Factor VII cDNA fragment from pUCVII1923; and a 2.7 kb Bam HI-Hind III fragment of pUC13. Three µg of FIX/VII-9 (RF DNA) were digested at 37°C for 6 hours with 45 units of Xba I in a volume of 50 µl. The DNA was precipitated with ethanol, resuspended and digested at 37°C for 4 h with 50 units of Hind III. The sample was subjected to electrophoresis in 1% agarose and the 0.6 kb band electro-eluted from the paper with 1.5 M NaCl, 50 mM Tris-HCl pH 8, 1 mM EDTA, phenol extracted and precipitated with ethanol.

To obtain the remaining Factor VII cDNA sequence, 5 µg of pUCVII1923 was digested at 37°C for 3 hours with 36 units of Xba I in 40 µl of medium salt buffer. Then 8 µl of 10x high salt buffer, 28 µl of H₂O, and 4 µl (40 units) of Bam HI were added and the reaction incubated at 37°C for 3 hours. The DNA fragments were separated by electrophoresis in 1% agarose and the 1.7 kb fragment isolated as described above.

The vector fragment was prepared by digesting 1 µg of pUC13 with 10 units of Hind III in 20 µl of medium salt buffer at 37°C for 1 hour. Two µl of 10x high salt buffer and 10 units of Bam HI were then added and the incubation continued for another 2 hours. The DNA was purified on a 1% agarose gel as described above.

Equimolar amounts (approximately 0.56 pmoles) of the three fragments were ligated at room temperature for 45 minutes in 10 µl of 50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 1 mM DTT, 1mM ATP and 3 units T₄ DNA ligase. The reaction mixture was used to transform competent *E. coli* JM83. The cells were plated on medium containing 40 µg/ml ampicillin with 50 µl of 2% X-gal added to each plate. DNA was prepared from 7 white colonies and then screened by restriction enzyme digestion. One of the clones giving the correct pattern was designated FIX/VII → pUC13.

Example 6: Expression of Biologically Active Factor VII Analogs.

The mammalian cell expression vector pD2 was chosen for expression of the FIX/VII gene in transfected animal cells. It was constructed from plasmid pDHFR-III (Berkner and Sharp, *Nuc. Acids Res.* 13: 841-857, 1985) in the following manner. The Pst I site abutting the DHFR cDNA in pDHFR III was

converted to a Bam HI site by conventional linking (Scheller, R.H., Dickerson, R.E., Boyer, H.W., Riggs, A.D., and Itakura, K., *Science* 196: 177-180, 1977). The pDHFR III DNA was incubated with 10 mM Tris pH 7.6, 6 mM β -MSH, 6 mM NaCl, 10 mM MgCl₂ and 2.5 units Pst I for 10 minutes at 37 °C, followed by phenol extraction and ethanol precipitation. The Pst I cohesive termini were blunt ended using T₄ DNA polymerase. After phenol extraction and dialysis against 10 mM Tris pH 8.0, 1 mM EDTA, 0.3 M NaCl, the DNA was ethanol precipitated. The DNA was resuspended in 20 μ l 1.4 mM ATP, 50 mM Tris pH 7.6, 10 mM MgCl₂, 1 mM dithiothreitol and then incubated with 5 ng of T₄ polynucleotide kinase-treated Bam HI linkers (New England Biolabs) and 200 units of T₄ polynucleotide ligase for 12 hours at 12 °C, followed by phenol extraction and ethanol precipitation. The DNA was digested with 90 units of Bam HI at 37 °C for 1 hour, followed by electrophoresis through a 1.4% agarose gel. The 4.9 kb DNA fragment (corresponding to pDHFR III DNA lacking the DHFR cDNA and SV40 polyadenylation signal) was electro-eluted and recircularized with polynucleotide ligase and then transfected into *E. coli* HB101. Ampicillin-sensitive colonies were screened by rapid prep analysis (Birnboim, H.C., and Doly, J., *Nucleic Acids Research* 7: 1513-1523, 1979) and the correct clone was grown up to generate a large-scale plasmid DNA preparation.

The resultant plasmid was cleaved with 20 units Bam HI and treated with 2.5 μ g calf intestinal phosphatase and electrophoresed on a 1.4% agarose gel. Twenty-five μ g of pSV40 (a clone of SV40 DNA inserted into the Bam HI site of pBR322) were digested with 25 units of Bcl I for 1 hour at 50 °C, followed by the addition of 25 units of Bam HI, and the incubation continued for 1 hour at 37 °C. This DNA was then electrophoresed on a 1.4% agarose gel. The Bam HI-cut vector (i.e., that lacking the polyadenylation signal) was joined to the SV40 DNA fragment (.14 to .19 map units [Tooze, J., ed., "DNA Tumor Viruses, Molecular Biology of Tumor Viruses"]) containing the late polyadenylation signal by incubating the gel-purified fragments (0.1 μ g each) in 20 μ l 50 mM Tris pH 7.6, 10 mM MgCl₂, 1 mM dithiothreitol, 1.4 mM ATP and 100 units T₄ polynucleotide ligase for 4 hours at 12 °C, followed by transformation into *E. coli* RR1. Positive colonies were identified by rapid prep analysis, and a large-scale plasmid preparation of the correct DNA, pD2, was prepared.

To make the Factor IX/VII expression construction, 1 μ g of pD2 was digested at 37 °C for 1 hour with 20 units of Bam HI in 20 μ l of high salt buffer. Twenty μ l of 10 mM Tris-HCl pH 8, 1 mM EDTA and 0.1 unit of calf alkaline phosphatase (Boehringer) were then added. The reaction was incubated at 37 °C for 1 hour and stopped by heating to 75 °C for 10 minutes. Ten μ g of FIX/VII \rightarrow pUC13 was digested at 37 °C for 2 hours with 150 units of Bam HI in 150 μ l of high salt buffer. The DNA fragments were separated by electrophoresis in 1.2% agarose and the 2.3 kb fragment was isolated. Equimolar amounts (0.015 pmoles) of the 2.3 kb Bam HI fragment and the pD2 vector fragment were ligated at 14 °C for 2.5 hours as above. The reaction mixture was used to transform *E. coli* RR1 cells, which were then plated on medium containing 10 μ g/ml ampicillin. Plasmid DNA was prepared from 12 of the resulting colonies and screened by restriction enzyme digestion. One of the clones with the correct enzyme digestion pattern was designated FIX/VII/pD2 (Figure 6). *E. coli* RR1 transformed with FIX/VII/pD2 has been deposited with ATCC under accession number 53068.

The procedure used to transfect baby hamster kidney (BHK) cells (available from American Type Culture Collection, accession number CCL10) with FIX/VII/pD2 was similar to published methods (for example, Wigler et al., *Cell* 14: 725, 1978; Corsaro and Pearson, *Somatic Cell Genetics* 7: 603, 1981; Graham and Van der Eb, *Virology* 52: 456, 1973). The BHK cells were grown at 37 °C, 5% CO₂, in Dulbecco's media (plus 10% heat-inactivated fetal calf serum and supplemented with glutamine and penicillin-strep-tomycin) in 60 mm tissue culture Petri dishes to a confluency of 20%. A total of 10 μ g DNA was used to transfect one 60 mm dish: 3.75 μ g of FIX/VII/pD2, 1.25 μ g of pKO-neo (Southern and Berg, J. Mol. Appl. Genet 1: 327-341, 1982) and 5 μ g of salmon sperm DNA. The DNAs were precipitated in 0.3 M NaOAc, 75% ethanol, rinsed with 70% ethanol and redissolved in 20 μ l 10 mM Tris-HCl pH 8, 1 mM EDTA. The DNA was combined with 440 μ l H₂O and 500 μ l of 280 mM NaCl, 1.5 mM NaHPO₄, 12 mM dextrose, 50 mM HEPES pH 7.12. Sixty μ l of 2 M CaCl₂ were added dropwise to the above mixture and the solution let stand at room temperature for 30 minutes. The solution was then added to the cells and the cells returned to 37 °C for 4 hours. The medium was removed and 5 ml of 20% DMSO in Dulbecco's with serum were added for 2 minutes at room temperature. The dish was then washed rapidly with 2 changes of medium and incubated in fresh medium overnight. Twenty-four hours after the DNA was added, the medium was removed and selective medium added (10 mg/ml of G418, 498 μ g/ml, Gibco, in Dulbecco's with serum). After 10 and 13 days, individual clones, representing cells that had incorporated the pKO-neo gene and were thus resistant to G418, were transferred to 96-well (or 24-well) plates and grown up for protein assays.

Cells were grown in Dulbecco's plus 10% fetal calf serum containing 5 μ g/ml vitamin K (Phytonadione, Merck). The medium was separated from the cells and cellular debris by centrifugation, and assayed for

Factor VII polypeptide (by ELISA) and for biological activity. The cells were removed from the plates with trypsin, washed with fresh medium, centrifuged, and frozen at -20 °C. The cell pellets were then thawed in PBS, pelleted, and resuspended in PBS containing 0.25% Triton X-100. Samples were diluted and assayed for polypeptide and activity.

5 The ELISA for Factor VII was done as follows. Two hundred microliters of a monoclonal antibody against human Factor VII (5 ul/ml in 0.1 M Na₂CO₃ pH 9.6) were incubated in each well of a 96-well microtiter plate 2 hours at 37 °C. The wells were then incubated with 220 ul of 1% bovine serum albumin (BSA) and 0.05% Tween 20 in PBS pH 7.2 2 hours at 37 °C. The plates were rinsed with H₂O, air dried, and stored at 4 °C. To assay samples, 200 ul samples were incubated 1 hour at room temperature in the
10 antibody-coated wells. The wells were then rinsed four times with 200 ul PBS containing 0.05% Tween 20. The wells were then incubated for 1 hour at room temperature with 200 ul of an IgG fraction of rabbit polyclonal antiserum against Factor VII (5 ug/ml in PBS containing 1% BSA and 0.05% Tween 20). This was followed by incubation with goat anti-rabbit IgG coupled to alkaline phosphatase. The wells were then
15 rinsed four times with PBS containing 0.05% Tween 20. To the wells were added 200 ul p-nitrophenyl phosphate (30 mg) dissolved in diethanolamine buffer (96 ml per liter) pH 9.8 containing 56 mg/l MgCl₂. The enzyme reaction was done at 37 °C and the development of a yellow color was monitored at 405 nm using an ELISA plate reader. Results obtained for cell media are given in Table 3.

Factor VII biological activity was assayed by the one-stage clotting assay described by Quick (Hemorrhagic Disease and Thrombosis, 2nd ed., Lea Febiger, Philadelphia, 1966). Results obtained for cell
20 media are given in Table 3.

<u>Day</u>	<u>Cells/ml (x10⁻⁴)</u>	<u>Factor VII polypeptide ng/ml</u>	<u>Factor VII activity (ng/ml)</u>
25			
1	2.9		
	2.7	25	6.0
30			
2	1.9		
	2.8	47	15.9
35			
3	1.96		
	2.26	160	93
40			
4	4.71		
	4.14	550	300
45			
5	8.79		
	11.28	725	531
50			
6	5.1		
	8.4	975	600

55 Example 7: Expression of Factor IX

Fourteen ug of FIX(-G) → pUC13 were digested with 30 units of Bam HI in 30 ul of high salt buffer for 3 hours at 37 °C. The DNA was then subjected to electrophoresis in 1% agarose and the 1.4 kb band

containing the Factor IX sequence was isolated from the gel.

Three ug of the vector pD2 were digested with 30 units of Bam HI in 30 ul high salt buffer for 3 hours at 37°C. The DNA was subjected to electrophoresis in 1% agarose and the linear 1.5 kb fragment isolated. The DNA was then treated with 0.12 units calf alkaline phosphatase in 30 ul of 10 mM Tris-HCl pH 8, 1 mM EDTA for 30 minutes at 37°C. The salt was adjusted to 0.3 M NaOAc and the sample extracted twice with phenol, once with chloroform and the DNA was ethanol precipitated. The pellet was rinsed in 70% ethanol, dried and redissolved in 20 ul 10 mM Tris-HCl pH 8, 1 mM EDTA. Equimolar amounts (0.02 pmoles) of the two fragments were ligated with 10 units of T₄ DNA ligase as described above. The reaction mixture was used to transform *E. coli* RR1 cells. DNA from twelve of the resulting ampicillin-resistant colonies was screened by restriction enzyme digestion. One of the clones with the 1.4 kb fragment inserted in the correct orientation was designated as FIX(-G)/pD2. *E. coli* RR1 transformed with FIX(-G)/pD2 has been deposited with ATCC under accession number 53067.

BHK cells were co-transfected with FIX(-G)/pD2 and pKO-neo as described above. Drug-resistant cells were selected and prepared for ELISA and activity assay as described in Example 6.

The assay for biological activity is based on the ability of Factor IX to reduce the clotting time of plasma from Factor IX-deficient patients to normal. It was done as described by Procter and Rapaport (Amer. J. Clin. Path. 36: 212, 1961). Results are shown in Table 4.

TABLE 4

Day	(x10 ⁻⁴)	Factor IX Cells/ml polypeptide (ng/ml)		Factor IX activity (ng/ml)	% active protein in
		supernatant	pellet	in supernatant	supernatant
1	1.65	-	-	-	-
2	2.66	57 45	20 20	27 24	50%
3	9.69	150 120	60 60	72 84	58%
4	14.79	475 225	160 140	198 150	50%
5	50.85	875 1000	250 260	408 438	45%

The amount of Factor IX polypeptide was determined by ELISA essentially as described in Example 6 using polyclonal rabbit antisera to Factor IX. Following the incubation of the wells with the Factor IX-containing samples, the wells were rinsed and incubated 1 hour at room temperature with 200 ul of affinity purified rabbit polyclonal anti-Factor IX conjugated to alkaline phosphatase diluted 1:1000 in PBS containing 1% BSA and 0.05% Tween 20. The wells were then rinsed four times with PBS containing 0.05% Tween 20, and enzyme substrate was added as above. Incubations were run at 4°C overnight or 37°C for 2 hours.

As shown in Table 4, 70-80% of the Factor IX polypeptide is secreted into the media, and about 50% of this is biologically active. No Factor IX activity was detected in the cell pellets.

Highest levels of activity were achieved by supplementing the cell culture medium with vitamin K (phytonadione, Merck) at concentrations of 1-10 mg/ml.

Several additional analyses were performed to demonstrate that the cells were secreting authentic Factor IX. Samples containing Factor IX activity according to the above assay were incubated with Factor VIII-deficient plasma but did not affect the clotting time, indicating that the activity was due to authentic Factor IX rather than a non-specific clotting agent. This conclusion was further verified by depletion of Factor IX activity from the samples with a specific antibody. Ninety-seven to ninety-eight percent of the Factor IX activity was immuno-precipitated from cell supernatants with a rabbit polyclonal antibody against Factor IX. This antibody also precipitated over 99% of the Factor IX activity from normal plasma. No Factor IX activity was removed from the supernatants by rabbit polyclonal antibody to erythropoietin.

Example 8: Construction of an expression vector for Factor VII.

An expression vector comprising the synthetic Factor VII 5' coding region joined to the partial Factor VII cDNA was constructed. The vector, designated pM7135, was generated by inserting the Factor IX leader - 5' Factor VII sequence from pM7115 and the 3' Factor VII sequence from FIX/VII/pD2 into plasmid pD3, which comprises the SV40 enhancer and the adenovirus 2 major late promoter and tripartite leader.

Plasmid pD3 was generated from plasmid pDHFR^{III}. The Pst I site immediately upstream from the DHFR sequence in pDHFR^{III} was converted to a Bcl I site by digesting 10 ug of plasmid with 5 units of Pst I for 10' at 37 °C in 100 ul buffer A (10 mM Tris pH 8, 10 mM MgCl₂, 6 mM NaCl, 7mM β-MSH). The DNA was phenol extracted, EtOH precipitated, and resuspended in 40 ul buffer B (50 mM Tris pH 8, 7 mM MgCl₂, 7 mM β-MSH) containing 10 mM dCTP and 16 units T4 DNA polymerase and incubated at 12 °C for 60 minutes. Following EtOH precipitation, the DNA was ligated to 2.5 ug kinased Bcl I linkers in 14 ul buffer C (10 mM Tris pH 8, 10 mM MgCl₂, 1 mM DTT, 1.4 mM ATP) containing 400 units T4 polynucleotide ligase for 12 hours at 12 °C. Following phenol extraction and EtOH precipitation, the DNA was resuspended in 120 ul buffer D (75 mM KCl, 6 mM Tris pH 7.5, 10 mM MgCl₂, 1 mM DTT), digested with 80 units Bcl I for 60 minutes at 50 °C, then electrophoresed through agarose. Form III plasmid DNA (10 ug) was isolated from the gel, and ligated in 10 ul buffer C containing 50 units T4 polynucleotide ligase for 2 hours at 12 °C, and used to transform *E. coli* HB101. Positive colonies were identified by rapid DNA preparation analysis, and plasmid DNA (designated pDHFR') prepared from positive colonies was transformed into dAM⁻ *E. coli*.

Plasmid pD2' was then generated by cleaving pDHFR' (15 ug) and pSV40 (25 ug) in 100 ul buffer D with 25 units Bcl I for 60 minutes at 50 °C, followed by the addition of 50 units Bam HI and additional incubation at 37 °C for 60 minutes. DNA fragments were resolved by agarose gel electrophoresis, and the 4.9 kb pDHFR' fragment and 0.2 kb SV40 fragment were isolated. These fragments (200 ng pDHFR' DNA and 100 ng SV40 DNA) were incubated in 10 ul buffer C containing 100 units T4 polynucleotide ligase for 4 hours at 12 °C, and the resulting construct (pD2') used to transform *E. coli* RRI.

Plasmid pD2' was modified by deleting the "poison" sequences in the pBR 322 region (Lusky and Botchan, Nature 293: 79-81, 1981). Plasmids pD2' (6.6 ug) and pML-1 (Lusky and Botchan, *ibid*) (4 ug) were incubated in 50 ul buffer A with 10 units each Eco RI and Nru I for 2 hours at 37 °C, followed by agarose gel electrophoresis. The 1.7 kb pD2' fragment and 1.8 kb pML-1 fragment were isolated and ligated together (50 ng each) in 20 ul buffer C containing 100 units T4 polynucleotide ligase for 2 hours at 12 °C, followed by transformation into *E. coli* HB101. Colonies containing the desired construct (designated Δ pD2) were identified by rapid preparation analysis. Ten ug of Δ pD2 were then digested with 20 units each Eco RI and Bgl II, in 50 ul buffer A for 2 hours at 37 °C. The DNA was electrophoresed through agarose, and the desired 2.8 kb fragment (fragment C) comprising the pBR322, 3' splice site and poly A sequences was isolated.

To generate the remaining fragments used in constructing pD3, pDHFR^{III} was modified to convert the Sac II (Sst II) site into either a Hind III or Kpn I site. Ten ug pDHFR^{III} were digested with 20 units Sst II for 2 hours at 37 °C, followed by phenol extraction and ethanol precipitation. Resuspended DNA was incubated in 100 ul buffer B containing 10 mM dCTP and 16 units T4 DNA polymerase for 60 minutes at 12 °C, phenol extracted, dialyzed, and ethanol precipitated. DNA (5 ug) was ligated with 50 ng kinased Hind III or Kpn I linkers in 20 ul buffer C containing 400 units T4 DNA ligase for 10 hours at 12 °C, phenol extracted, and ethanol precipitated. After resuspension in 50 ul buffer A, the resultant plasmids were digested with 50 units Hind III or Kpn I, as appropriate, and electrophoresed through agarose. Gel-isolated DNA (250 ng) was ligated in 30 ul buffer C containing 400 units T4 DNA ligase for 4 hours at 12 °C and used to transform *E. coli* RRI. The resultant plasmids were designated pDHFR^{III} (Hind III) and pDHFR^{III} (Kpn I). A 700 bp Kpn I-Bgl II fragment (fragment A) was then purified from pDHFR^{III} (Kpn I) by digestion with Bgl II and Kpn I followed by agarose gel electrophoresis.

The SV40 enhancer sequence was inserted into pDHFR^{III} (Hind III) as follows: 50 ug SV40 DNA was incubated in 120 ul buffer A with 50 units Hind III for 2 hours at 37 °C, and the Hind III C SV40 fragment

(5089-968 bp) was gel purified. Plasmid pDHFRIII (Hind III) (10 ug) was treated with 250 ng calf intestinal phosphatase for 1 hour at 37°C, phenol extracted and ethanol precipitated. The linearized plasmid (50 ng) was ligated with 250 ng Hind III C SV40 in 16 ul buffer C for 3 hours at 12°C, using 200 units T4 polynucleotide ligase, and transformed into *E. coli* HB101. A 700 base pair Eco RI-Kpn I fragment (fragment B) was then isolated from this plasmid.

For the final construction of pD3, fragments A and B (50 ng each) were ligated with 10 ng fragment C with 200 units T4 polynucleotide ligase for 4 hours at 12°C, followed by transfection of *E. coli* RRI. Positive colonies were detected by rapid preparation analysis, and a large-scale preparation of pD3 was made.

Expression vector pM7135 was then constructed. The replicative form of pM7115 was digested with Bam HI and Xba I and the 550 base pair fragment comprising the Factor IX leader and 5' Factor VII sequence was gel purified. Plasmid FIX/VII/pD2 was digested with Xba I and Bam HI and the 1700 bp fragment comprising the 3' portion of the Factor VII cDNA was gel purified. Plasmid pD3 was digested with Bcl I, treated with calf alkaline phosphatase, and the three fragments joined in a triple ligation. The resultant constructs were screened for the presence of a 2000 base pair Xba I fragment. A plasmid having the correct orientation was selected and designated pM7135 (Figure 8).

Example 9: Expression of Factor VII From cDNA Clones.

In order to express Factor VII cDNA containing a Factor VII leader, DNA from λ VII2463 or λ VII565 and λ VII2463 was cloned into an expression vector containing the Ad2 major late promoter, SV40 enhancer sequences, the Ad2 tripartite leader, a splice set, and the SV40 polyadenylation signal. This vector was adapted so that it contains a unique EcoRI sequence as the site of cDNA insertion. The expression of sequences from λ VII2463, which encodes a 60 amino acid leader, and from λ VII565 and λ VII2463, which lacks the codons for amino acids from -18 to -39 and thus encodes a leader 38 amino acids in length, were evaluated. Because the structure of the Factor VII leader has only been identified by cDNA cloning, and because of the ambiguity generated by having obtained two different 5' -terminal cDNAs, the inventors also constructed a genomic-cDNA Factor VII sequence. The 3' portion of λ VII2463 (from the Bgl II site in exon 2, to the EcoRI site linked 3' to the poly(A) tail) was adjoined to a subgenomic fragment of clone 7m1, that encoding exons 1a, 1b and the remainder of exon 2. This subgenomic fragment, reconstructed as an EcoRI-BglII 4.4 Kb fragment, was adjoined to the λ VII2463 cDNA and cloned into a mammalian expression vector.

Briefly, in order to construct the subclones, the Factor VII cDNA EcoRI fragment of λ VII2463 was cloned into the EcoRI site of pUC18, and designated pVII2463. Similarly, the EcoRI cDNA insert of λ VII565 was subcloned into pUC18, and designated pVII565. A hybrid between the 5' portion of the Factor VII sequence of clone pVII565 and the 3' segment of Factor VII DNA of pVII2463 was constructed by cloning the 5'-most EcoRI-Bgl II Factor VII fragment of pVII565 and the Bgl II-Hind III Factor VII fragment (Hind III site in polylinker of pVII2463) of pVII2463 into pUC 18 digested with EcoRI and Hind III. This construct was designated pVII2397. The inserts of pVII2463 and pVII2397 were removed by EcoRI digestion and gel purified for insertion into mammalian expression vectors as described below.

A. Expression of full-length Factor VII cDNA.

The expression of Factor VII was achieved in the vector pDX. This vector was derived from pD3 (described in Example 8 above) and pD3', a vector identical to pD3 except that the SV40 polyadenylation signal (i.e. the SV40 Bam HI [2533 bp] to BclI [2770bp] fragment) is in the late orientation. Thus, pD3' contains a Bam HI site as the site of gene insertion.

To generate pDX, the EcoRI site in pD3' was converted to a Bcl I site by Eco RI cleavage, incubation with S1 nuclease, and subsequent ligation with Bcl I linkers. DNA was prepared from a positively identified colony, and the 1.9 kb XhoI-PstI fragment containing the altered restriction site was prepared via agarose gel electrophoresis. In a second modification, Bcl I-cleaved pD3 was ligated with kinased Eco RI-Bcl I adaptors (constructed from oligonucleotides ZC 525, 5'GGAATTCT³; and ZC526, 5'GATCAGAATTCC³) in order to generate an Eco RI site as the position for inserting a gene into the expression vector. Positive colonies were identified by restriction endonuclease analysis, and DNA from this was used to isolate a 2.3 kb XhoI-PstI fragment containing the modified restriction site. The two above-described DNA fragments were incubated together with T4 DNA ligase, transformed into *E. coli* HB101 and positive colonies were identified by restriction analysis. A preparation of such DNA, termed pDX, was made (Figure 12). This DNA was cleaved with Eco RI and subsequently incubated with calf-intestinal phosphatase. The purified DNA was then incubated with T4 DNA ligase and the Factor VII Eco RI fragment from pVII2463, or with the Factor VII Eco RI cDNA fragment derived from pVII2397. The resultant clones were designated FVII(2463)/pDX and

FVII(565+2463)/pDX, respectively (Figure 12). After transformation into *E. coli* JM83 and subsequent identification by restriction enzyme analysis, plasmid DNA preparations were made and checked by extensive restriction endonuclease digestion. The plasmids FVII(2463)/pDX and FVII(565 + 2463)/pDX have been deposited with American Type Culture Collection and have been assigned accession numbers 40206; and 40205, respectively.

FVII(2463)/pDX and FVII(565 + 2463)/pDX (10 ug each) were each transfected, along with 10 ug salmon sperm carrier DNA, into either BHK tk⁻ts13 cells (Floros et al., *Exper. Cell Res.* 132: 215-223, 1981) or COS cells, using standard calcium-phosphate precipitation. Following transfection, the cells were cultured in the appropriate media containing 5 ug/ml vitamin K for two days. At this time, the supernatants were assayed for ELISA-positive material, using a monoclonal antibody directed against Factor VII. Both FVII(2463)/pDX and FVII(565 + 2463)/pDX directed the production of Factor VII polypeptide which was detected in COS cell supernatants, and Factor VII from FVII(565 + 2463)/pDX was detected in BHK cell supernatant. Sham-transfected BHK cells or COS cells did not yield detectable levels of Factor VII (Table 5).

TABLE 5

20	DNA	Cell Line	Cell Number	ELISA Positive Material (ng/ml Culture Medium)
	FVII(2463)/pDX	COS	2 x 10 ⁶	15
	FVII(565+2463)/pDX	COS	2 x 10 ⁶	12
25	Control	COS	2 x 10 ⁶	<2
	FVII(2463)/pDX	BHK	9 x 10 ⁶	62
	FVII(565+2463)/pDX	BHK	9 x 10 ⁶	6
30	Control	BHK	9 x 10 ⁶	<2

Transient expression of Factor VII and also tested in several other cell lines, listed in Table 6.

TABLE 6

<u>Name</u>	<u>Description</u>	<u>Reference (ATTC #)</u>
1. Rat Hep I	Rat hepatoma H4-II-E-C3	CRL 1600
2. Rat Hep II	Rat hepatoma H4-II-E	CRL 1548
3. TCMK	Mouse Kidney, SV40 virus transformed, TCMK-1	CCL 139
4. Human lung	SV40 virus transformed WI-38 VA13, subline 2RA	CCL 75.1
5. Human hepatoma	Human liver adenocarcinoma SK-HEP-1	HTB-52
6. Hep G2	Human hepatoma, dev. by Barbara Knowles/Wistar Institute	HTB 8065
7. Mouse liver	NCTC clone 1469	CC 29.1
8. COS	SV40-transformed CV-1 (monkey) cells	CRL 1650
9. BHK	Baby hamster kidney BHK-21 (C-13)	CCL 10
10. 293	Human embryonic Kidney/Ad transformed	CRL 1573
11. DUKX	CHO-DHFR ^{sens}	(Urlaub & Chasin, PNAS (USA) 77: 4216-4220, 1980)

Cells were cotransfected with 10 ug of either FVII(2463)/pDX or FVII(565 + 2463)/pDX together with 1 ug of a plasmid comprising the chloramphenicol acetyl transacetylase gene (to permit identification of cotransfected cells) and 10 ug of salmon sperm DNA. Mock transfected cells were used as controls. Spent media were assayed by ELISA after six days. Results are given in Table 7.

TABLE 7

	<u>Sample</u>	<u>Cell Line</u>	<u>Plasmid</u>	<u>Cell Number x 10⁻⁶</u>	<u>ELISA (ng/ml)</u>
5	1.	Rat Hep I	Mock	11.6	2.4
10	2.	Rat Hep I	FVII(565+2463)/pDX	7.0	2
	3.	Rat Hep I	FVII(2463)/pDX	7.0	<2
	4.	Rat Hep 2	Mock	13.0	<2
15	5.	Rat Hep 2	FVII(565+2463)/pDX	18.4	<2
	6.	Rat Hep 2	FVII(2463)/pDX	14.6	<2
20	7.	TCMK	Mock	18.8	<2
	8.	TCMK	FVII(565+2463)/pDX	9.8	<2
	9.	TCMK	FVII(2463)/pDX	12.8	<2
25	10.	Human Lung	Mock	7.2	<2
	11.	Human Lung	FVII(565+2463)/pDX	3.4	16.5

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	12.	Human Lung	FVII(2463)/pDX	3.4	12.2
5	13.	Human Hepatoma	Mock	13.0	<2
	14.	Human Hepatoma	FVII(565+2463)/pDX	11.0	3.5
	15.	Human Hepatoma	FVII(2463)/pDX	6.0	3.0
10	16.	HepG2	Mock	6.8	21
	17.	HepG2	FVII(565+2463)/pDX	6.0	45.5
15	18.	HepG2	FVII(2463)/pDX	6.0	28
	19.	Mouse Liver	Mock	3.8	<2
	20.	Mouse Liver	FVII(565+2463)/pDX	4.0	<2
20	21.	Mouse Liver	FVII(2463)/pDX	3.6	<2
	22.	COS	Mock	5.6	<2
25	23.	COS	FVII(565+2463)/pDX	5.6	15.5
	24.	COS	FVII(2463)/pDX	4.4	14.5
	25.	BHK tk ^S -ts13	Mock	3.0	<2
30	26.	BHK tk ^S -ts13	FVII(565+2463)/pDX	5.0	25
	27.	BKH tk ^S -ts13	FVII(2463)/pDX	4.0	22.5
35	28.	293	Mock	5.8	<2
	29.	293	FVII(565+2463)/pDX	6.2	94
40	30.	293	FVII(2463)/pDX	8.2	100
	31.	DUKX	Mock	11.6	<2
	32.	DUKX	FVII(565+2463)/pDX	13.0	<2
45	33.	DUKX	FVII(2463)/pDX	13.6	<2

FVII(2463)/pDX (10 ug) or FVII(565+2463)/pDX (10 ug) was co-transfected with 10 ug of salmon sperm DNA and 1 ug of a plasmid encoding the resistant form of dihydrofolate reductase (Simonsen and Levinson, Proc. Natl. Acad. Sci. USA 80: 2495-2499, 1983) in a mammalian expression vector, into BHK tk^S-ts13 cells. After two days, the cells were split 1:14 and placed into selective media containing either 250 nM or 1000 nM methotrexate (MTX) and 5 ug/ml vitamin K (phytadione, Merck). After two weeks, colonies were isolated and grown to 50-90% confluency. The supernatant media were then assayed for Factor VII polypeptide by ELISA. Of the 25 positive clones, 22 were analyzed further. The cells were plated at 5×10^4 (Group I) or 1×10^5 (Group II) in 10 cm dishes containing 5 ug/ml vitamin K, and either 250 nM or 1000 nM methotrexate. Five days later, the faster growing clones (designated by an asterisk in Table 8) were split 1:2, then after 24 hours, the media were changed on all plates. Twenty-three hours (Group I) or 20 hours (Group II) later,

supernatant media were harvested and cell counts were taken for each clone. The media were assayed both by ELISA and by the one-stage clotting assay. Results are shown in Table 8.

TABLE 8
GROUP I - 23-hour assay

Clone	Plasmid	Cell Count $\times 10^{-5}$	ELISA (pg/ cell/ day)	ELISA (ng/ ml)	Clot- ting (ng/ ml)	% Active
B4-A1*	FVII(565+2463)/pDX	2	6.5	130	206	158
B4-B1*	FVII(565+2463)/pDX	27	1.9	513	360	70
B4-C1	FVII(565+2463)/pDX	16	2.5	393	480	122
B4-C2*	FVII(565+2463)/pDX	9	<0.2	<20	21	-
B4-C3	FVII(565+2463)/pDX	52	1.5	800	910	114
B4-D1*	FVII(565+2463)/pDX	27	2.0	553	570	103
B4-D2*	FVII(565+2463)/pDX	13	1.2	150	154	103
B4-E1	FVII(565+2463)/pDX	39	2.2	870	1160	133
B4-E2*	FVII(565+2463)/pDX	8	2.5	205	240	117
B4-E3	FVII(565+2463)/pDX	23	1.2	275	320	116
B4-E4	FVII(565+2463)/pDX	31	1.3	410	300	73
B3-5.3*	FVII(2463)/pDX	5	8.2	410	290	70

GROUP II - 20-hour assay

Clone	Plasmid	Cell Count $\times 10^{-5}$	ELISA (pg/ cell/ day)	ELISA (ng/ ml)	Clot- ting (ng/ ml)	% Active
B3-2.2	FVII(2463)/pDX	41	2.5	1043	500	48
B3-2.3	FVII(2463)/pDX	19	3.0	580	610	105
B3-3.2	FVII(2463)/pDX	13	1.5	197	216	110
B3-4.2	FVII(2463)/pDX	41	1.8	760	620	82
B3-5.1	FVII(2463)/pDX	14	3.3	460	400	87
B3-5.2	FVII(2463)/pDX	9	2.7	257	184	72
B6-D	FVII(2463)/pDX	54	3.0	1700	780	46
B6-E	FVII(2463)/pDX	101	1.6	1640	970	59
B6-G	FVII(2463)/pDX	31	5.7	1853	1080	58
B6-M	FVII(2463)/pDX	75	2.2	1743	940	54

B. Expression of Factor VII genomic-cDNA hybrid.

An expression vector containing genomic sequences representing the Factor VII genomic 5'-terminus and cDNA sequences from the Factor VII gene 3'-terminus was prepared as follows. Three subclones of the genomic plasmid 7 m1 were used to reconstruct the 5'-terminus: 7Bam, 7SD and 7SE. Plasmid 7Bam is a 3.6 Kb EcoRI-Bam HI fragment containing exon 1a, subcloned into pUC12. A 0.7 Kb EcoRI-XbaI fragment, which contains exon 1a, was isolated from this subclone and is designated fragment a. Plasmid 7SD is a 3.7 Kb SstI fragment containing exon 1b, subcloned into pUC18. An exon 1b-containing 3.1 Kb XbaI-SstI fragment was isolated from this subclone and is designated fragment b. Plasmid 7SE is a 3.9 Kb SstI fragment containing exons 2-4, subcloned into M13mp 19. An SstI-Bgl II (0.6 Kb) fragment containing the 5' part of exon 2 was gel isolated and is designated fragment c. The remainder of the 3'-Factor VII cDNA (fragment d) was obtained as a 2 Kb BglII-EcoRI fragment from pUCVII2463. Fragments a-d were ligated with EcoRI-cleaved and calf intestinal-phosphatased pDX, and then transformed into *E. coli* JM83 or HB101. Positive colonies were identified by restriction endonuclease analysis, and plasmid DNA was prepared from these colonies.

For expression of Factor VII, the plasmid DNA is co-transfected into BHK or COS cells as described above. Transfected cells are cultured in vitamin K-containing medium for 2 days, and the medium is assayed for Factor VII by ELISA.

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made. Accordingly, the invention is not to be limited except by the appended claims.

The various strains of *E. Coli* used in the foregoing Examples were the personal choice of the inventors. Persons skilled in this art will appreciate that other suitable strains of *E. Coli* could be substituted (for example if the person already has in his possession samples of other suitable *E. Coli* strains).

Transformants ATCC 53067 and 53068 were deposited with the American Type Culture Collection on 28 March 1985 and plasmids ATCC 40205 and 40206 were deposited with the American Type Culture Collection on 25 November 1985, all four deposits being made in accordance with the Budapest Treaty.

The features disclosed in the foregoing description, in the following claims and/or in the accompanying drawings may, both separately and in any combination thereof, be material for realising the invention in diverse forms thereof.

Claims

1. A DNA construct containing a nucleotide sequence encoding human factor VII having an amino acid sequence as shown in Figure 1b.
2. The DNA construct of Claim 1 wherein said nucleotide sequence also encodes a leader peptide.
3. The DNA construct of Claim 1 wherein said nucleotide sequence includes a synthesized double-stranded oligonucleotide.
4. The DNA construct of Claim 3 wherein said synthesized double-stranded oligonucleotide codes for the amino-terminal portion of Factor VII.
5. The DNA construct of Claim 1 wherein at least a portion of said nucleotide sequence is derived from a cDNA clone or a genomic clone of Factor VII.
6. The DNA construct of Claim 1 comprising a first nucleotide sequence derived from a cDNA or a genomic clone of Factor VII, joined to a second nucleotide sequence positioned downstream of said first sequence, said second sequence derived from a cDNA clone of Factor VII, the joined sequences coding for a protein which upon activation has Factor VIIa biological activity for blood coagulation.
7. The DNA construct of Claim 1 wherein said nucleotide sequence comprises the cDNA sequence of Figure 1b, from bp 36 to bp 1433.
8. The DNA construct of Claim 1 wherein said nucleotide sequence comprises the cDNA sequence of Figure 1b, from bp 36 to bp 99, followed downstream by the sequence from bp 166 to bp 1433.

9. The DNA construct of Claim 1 wherein said nucleotide sequence codes for the amino acid sequence of Figure 1b from alanine, amino acid number 1, to proline, amino acid number 406.
10. A recombinant plasmid capable of integration in mammalian host cell DNA, said plasmid including a promoter followed downstream by a nucleotide sequence according to any of the previous Claims 1 to 9, said nucleotide sequence being followed downstream by a polyadenylation signal.
11. Mammalian cells transfected with a recombinant plasmid according to Claim 10.
12. A method for producing a protein having biological activity for blood coagulation mediated by Factor VIIa, comprising:
 - establishing a mammalian host cell which contains a DNA construct containing a nucleotide sequence encoding human factor VII;
 - growing said mammalian host cell in an appropriate medium which contains vitamin K;
 - isolating the protein product encoded by said DNA construct produced by said mammalian host cell; and
 - activating said protein product to generate a protein which has Factor VIIa biological activity for blood coagulation.
13. The method of Claim 12, including amplification of the DNA construct by cotransfection of the host cell with a gene encoding dihydrofolate reductase, wherein the appropriate medium comprises methotrexate.
14. The method of Claim 12 wherein said protein product is activated by reacting the protein with a proteolytic enzyme selected from the group consisting of Factor XIIIa, Factor IXa, kallikrein, Factor Xa, and thrombin.
15. A pharmaceutical preparation for the treatment of bleeding disorders containing a protein having an amino acid sequence as shown in Figure 1b and free of contaminating human proteins.
16. A method of producing a protein having biological activity for blood coagulation mediated by Factor VIIa, which process comprises growing in an appropriate medium which contains vitamin K an established mammalian host cell which contains a DNA construct containing a nucleotide sequence encoding human factor VII, isolating the protein product encoded by said DNA construct produced by said mammalian host cell and activating said protein product to generate a protein which has Factor VIIa biological activity for blood coagulation.
17. The method of Claim 12, 13, 14 or 16 wherein said host cell is a non-hepatic cell.
18. A method of preparing a pharmaceutical composition for the treatment of bleeding disorders, which method comprises preparing a pharmaceutical composition containing a protein produced by the method of any one of Claims 12, 13, 14, 16 and 17.

Patentansprüche

1. DNA-Aufbau, der eine Nukleotidsequenz enthält, welche menschlichen Faktor VII kodiert, mit einer Aminosäuresequenz wie in Figur 1b gezeigt.
2. DNA-Aufbau nach Anspruch 1, bei dem die Nukleotidsequenz auch ein Leader-Peptid kodiert.
3. DNA-Aufbau nach Anspruch 1, bei dem die Nukleotidsequenz ein synthetisiertes doppelsträngiges Oligonukleotid umfaßt.
4. DNA-Aufbau nach Anspruch 3, bei dem das synthetisierte doppelsträngige Oligonukleotid für den Aminoende-Abschnitt des Faktors VII kodiert.
5. DNA-Aufbau nach Anspruch 1, bei dem wenigstens ein Abschnitt der Nukleotidsequenz von einem cDNA-Klon oder einem genomischen Klon des Faktors VII abgeleitet ist.

6. DNA-Aufbau nach Anspruch 1, welcher eine erste Nukleotidsequenz aufweist, die von einem cDNA- oder einem genomischen Klon des Faktors VII abgeleitet ist, angebunden an eine zweite Nukleotidsequenz, die stromabwärts der ersten Sequenz angeordnet ist, wobei die zweite Sequenz von einem cDNA-Klon des Faktors VII abgeleitet ist, wobei die verbundenen Sequenzen für ein Protein kodieren, das nach der Aktivierung die biologische Aktivität des Faktors VIIa für Blutgerinnung hat.
7. DNA-Aufbau nach Anspruch 1, bei dem die Nukleotidsequenz eine cDNA-Sequenz der Figur 1b umfaßt, von Basenpaar 36 bis Basenpaar 1433.
8. DNA-Aufbau nach Anspruch 1, bei dem die Nukleotidsequenz die cDNA-Sequenz der Figur 1b aufweist, von Basenpaar 36 bis Basenpaar 99, stromaufwärts gefolgt von der Sequenz von Basenpaar 166 bis Basenpaar 1433.
9. DNA-Aufbau nach Anspruch 1, bei dem die Nukleotidsequenz für die Aminosäuresequenz der Figur 1b von Alanin, Aminosäure Nr. 1, bis Prolin, Aminosäure Nr. 406 kodiert.
10. Rekombinantes Plasmid, geeignet zur Integration in Säugetier-Wirtszellen-DNA, wobei das Plasmid einen Promotor umfaßt, der stromabwärts von einer Nukleotidsequenz gemäß einem der vorangehenden Ansprüche 1 bis 9 gefolgt ist, wobei die Nukleotidsequenz stromabwärts von einem Polyadenilierungssignal gefolgt ist.
11. Säugetierzellen, transfiziert mit einem rekombinanten Plasmid nach Anspruch 10.
12. Verfahren zum Produzieren eines Proteins mit biologischer Aktivität für Blutgerinnung, vermittelt durch Faktor VIIa, umfassend:
Einrichten einer Säugetier-Wirtszelle, welche einen DNA-Aufbau enthält, der eine menschlichen Faktor VII kodierende Nukleotidsequenz enthält;
Züchten der Säugetier-Wirtszelle in einem geeigneten Medium, welches Vitamin K enthält;
Isolieren des Proteinproduktes, kodiert von dem DNA-Aufbau, das von der Säugetier-Wirtszelle produziert worden ist; und
Aktivieren des Proteinproduktes, um ein Protein zu erzeugen, das eine biologische Aktivität für Blutgerinnung von Faktor VIIa hat.
13. Verfahren nach Anspruch 12, einschließlich der Verstärkung des DNA-Aufbaus durch Kotransfektion der Wirtszelle mit einem Gen, das die Hydrofolat-Reduktase kodiert, wobei das geeignete Medium Methotrexat aufweist.
14. Verfahren nach Anspruch 12, bei dem das Proteinprodukt aktiviert wird, indem das Protein mit einem proteolytischen Enzym reagiert wird, das aus der Gruppe bestehend aus Faktor XIIa, Faktor IXa, Kalikrein, Faktor Xa und Thrombin ausgewählt ist.
15. Pharmazeutische Präparation für die Behandlung von Blutungserkrankungen, welche ein Protein mit einer Aminosäuresequenz wie in Figur 1b gezeigt und frei von kontaminierenden menschlichen Proteinen enthält.
16. Verfahren zum Produzieren eines Proteins mit biologischer Aktivität für Blutgerinnung, vermittelt durch Faktor VIIa, bei dem der Prozeß das Züchten einer eingerichteten Säugetier-Wirtszelle, welche einen DNA-Aufbau, der eine menschlichen Faktor VII kodierende Nukleotidsequenz enthält, enthält, in einem geeigneten Medium, das Vitamin K enthält, das Isolieren des von diesem DNA-Aufbau kodierten Proteinproduktes, das von der Säugetier-Wirtszelle produziert worden ist, und das Aktivieren des Proteinproduktes, um ein Protein zu erzeugen, das die biologische Aktivität für Blutgerinnung von Faktor VIIa hat, aufweist.
17. Verfahren nach Anspruch 12, 13, 14 oder 16, bei dem die Zelle eine nicht-hepatische Zelle ist.
18. Verfahren zum Präparieren einer pharmazeutischen Zusammensetzung für die Behandlung von Blutungserkrankungen, wobei das Verfahren das Präparieren einer pharmazeutischen Zusammensetzung umfaßt, die ein durch das Verfahren nach einem der Ansprüche 12, 13, 14, 16 und 17 produziertes

Protein enthält.

Revendications

- 5 1. Produit de recombinaison d'ADN contenant une séquence de nucléotides codant pour le facteur humain VII, ayant une séquence d'acides-amino telle que représentée sur la figure 1b.
2. Produit de recombinaison d'ADN suivant la revendication 1, dans lequel la séquence de nucléotides code aussi pour un peptide leader.
- 10 3. Produit de recombinaison d'ADN suivant la revendication 1, dans lequel la séquence de nucléotides renferme un oligonucléotide double brin synthétisé.
4. Produit de recombinaison d'ADN suivant la revendication 3, dans lequel l'oligonucléotide double brin synthétisé code pour la portion terminale amino du facteur VII.
- 15 5. Produit de recombinaison d'ADN suivant la revendication 1, dans lequel au moins une portion de la séquence de nucléotides est dérivée d'un clone d'ADNc ou d'un clone génomique du facteur VII.
- 20 6. Produit de recombinaison d'ADN suivant la revendication 1, comprenant une première séquence de nucléotides dérivée d'un clone d'ADNc ou d'un clone génomique du facteur VII, liée à une seconde séquence de nucléotides disposée en aval de la première séquence, la seconde séquence étant dérivée d'un clone d'ADNc du facteur VII, les séquences liées codant pour une protéine qui, par activation, possède l'activité biologique du facteur VIIa pour la coagulation du sang.
- 25 7. Produit de recombinaison d'ADN suivant la revendication 1, dans lequel la séquence de nucléotides comprend la séquence d'ADNc de la figure 1b, de la paire de bases 36 à la paire de bases 1433.
8. Produit de recombinaison d'ADN suivant la revendication 1, dans lequel la séquence de nucléotides comprend la séquence d'ADNc de la figure 1b, de la paire de bases 36 à la paire de bases 99, suivie en aval de la séquence allant de la paire de bases 166 à la paire de bases 1433.
- 30 9. Produit de recombinaison d'ADN suivant la revendication 1, dans lequel la séquence de nucléotides code pour la séquence d'acides-amino de la figure 1b allant de l'acide-amino, alanine, numéro 1 à l'acide-amino, proline, numéro 406.
- 35 10. Plasmide recombinant susceptible d'intégration dans l'ADN d'une cellule-hôte de mammifère, ledit plasmide comprenant un promoteur suivi en aval d'une séquence de nucléotides suivant l'une quelconque des revendications 1 à 9, ladite séquence de nucléotides étant suivie en aval d'un signal de polyadénylation.
- 40 11. Cellules de mammifère transfectées avec un plasmide recombinant suivant la revendication 10.
12. Procédé de production d'une protéine douée d'activité biologique pour la coagulation du sang sous la médiation du facteur VIIa, comprenant :
l'établissement d'une cellule-hôte de mammifère qui contient un produit de recombinaison d'ADN comportant une séquence de nucléotides codant pour le facteur humain VII ;
la croissance de ladite cellule-hôte de mammifère dans un milieu approprié qui contient de la vitamine K ;
50 l'isolement du produit protéique codé par le produit de recombinaison d'ADN élaboré par la cellule-hôte de mammifère ; et
l'activation dudit produit protéique pour engendrer une protéine qui a l'activité biologique du facteur VIIa pour la coagulation du sang.
- 55 13. Procédé suivant la revendication 12, comportant l'amplification du produit de recombinaison d'ADN par co-transfection de la cellule-hôte avec un gène codant pour la dihydrofolate-réductase, où le milieu approprié comprend du méthotrexate.

14. Procédé suivant la revendication 12, dans lequel le produit protéique est activé par réaction de la protéine avec un enzyme protéolytique choisi dans le groupe comprenant le facteur XIIa, le facteur IXa, la kallikréine, le facteur Xa et la thrombine.
- 5 15. Préparation pharmaceutique destinée au traitement de troubles hémorragiques, contenant une protéine ayant une séquence d'acides-amino telle que représentée sur la figure 1b et dépourvue de protéines humaines contaminantes.
- 10 16. Procédé de production d'une protéine douée d'activité biologique pour la coagulation du sang sous la médiation du facteur VIIa, procédé qui comprend la croissance, dans un milieu approprié contenant de la vitamine K, d'une cellule-hôte de mammifère établie qui contient un produit de recombinaison d'ADN contenant une séquence de nucléotides codant pour le facteur humain VII, l'isolement du produit protéique codé par le produit de recombinaison d'ADN élaboré par ladite cellule-hôte de mammifère et l'activation du produit protéique pour engendrer une protéine qui a l'activité biologique du facteur VIIa
15 pour la coagulation du sang.
17. Procédé suivant la revendication 12, 13, 14 ou 16, dans lequel la cellule-hôte est une cellule non hépatique.
- 20 18. Procédé de préparation d'une composition destinée au traitement de troubles hémorragiques, procédé qui comprend la préparation d'une composition pharmaceutique contenant une protéine produite par le procédé suivant l'une quelconque des revendications 12, 13, 14, 16 et 17.

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FIG. 1A

EcoRIa 24 39 54
 GAATTCGG TGC AGG ACG AAG CTG TTC TGG ATT TCT TAC AGT GAT GGG GAC CAG
 Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp Gln

69 84 99
 TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG CTC CAG TCC
 Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln Leu Gln Ser

114 129 144 159
 TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC TGT GAG ACG CAC AAG
 Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn Cys Glu Thr His Lys

174 189 204 Pst Ia
 GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC GGC TGT GAG CAG TAC TGC AGT
 Asp Asp Gln Leu Ile Cys Val Asn Glu Asp Gly Gly Cys Glu Gln Tyr Cys Ser

219 234 249 264
 GAC CAC ACG GGC ACC AAG CGC TCC TGT CGG TGC CAC GAG GGG TAC TCT CTG CTG
 Asp His Thr Gly Thr Lys Arg Ser Cys Arg Cys His Glu Gly Tyr Ser Leu Leu

279 294 309 324
 GCA GAC GGG GTG TCC TGC ACA CCC ACA GTT GAA TAT CCA TGT GGA AAA ATA CCT
 Ala Asp Gly Val Ser Cys Thr Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro

Xba I 339 354 369
 ATT CTA GAA AAA AGA AAT GCC AGC AAA CCC CAA GGC CGA ATT GTG GGG GGC AAG
 Ile Leu Glu Lys Arg Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys

384 399 414 429
 GTG TGC CCC AAA GGG GAG TGT CCA TGG CAG GTC CTG TTG TTG GTG AAT GGA GCT
 Val Cys Pro Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala

444 459 474
 CAG TTG TGT GGG GGG ACC CTG ATC AAC ACC ATC TGG GTG GTC TCC GCG GCC CAC
 Gln Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala His

489 504 519 534
 TGT TTC GAC AAA ATC AAG AAC TGG AGG AAC CTG ATC GCG GTG CTG GGC GAG CAC
 Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu Gly Glu His

549 564 579 594
 GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGG CGG GTG GCG CAG GTC ATC
 Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg Val Ala Gln Val Ile

609 Sma I 624 639
 ATC CCC AGC ACG TAC GTC CCG GGC ACC ACC AAC CAC GAC ATC GCG CTG CTC CGC
 Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn His Asp Ile Ala Leu Leu Arg

654 669 684 699
 CTG CAC CAG CCC GTG GTC CTC ACT GAC CAT GTG GTG CCC CTC TGC CTG CCC GAA
 Leu His Gln Pro Val Val Leu Thr Asp His Val Val Pro Leu Cys Leu Pro Glu

714 729 744
 CGG ACG TTC TCT GAG AGG ACG CTG GCC TTC GTG CGC TTC TCA TTG GTC AGC GGC
 Arg Thr Phe Ser Glu Arg Thr Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly

759 774 Nar I 789 804
 TGG GGC CAG CTG CTG GAC CGT GGC GCC ACG GCC CTG GAG CTC ATG GTC CTC AAC
 Trp Gly Gln Leu Leu Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn

819 834 Pst Ib 849 864
 GTG CCC CGG CTG ATG ACC CAG GAC TGC CTG CAG CAG TCA CGG AAG GTG GGA GAC
 Val Pro Arg Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp

879 894 909
 TCC CCA AAT ATC ACG GAG TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC AAG
 Ser Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys

924 939 954 969
 GAC TCC TGC AAG GGG GAC AGT GGA GGC CCA CAT GCC ACC CAC TAC CGG GGC ACG
 Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg Gly Thr

984 999 1014
 TGG TAC CTG ACG GGC ATC GTC AGC TGG GGC CAG GGC TGC GCA ACC GTG GGC CAC
 Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala Thr Val Gly His

1029 1044 1059 TaqI 1074
 TTT GGG GTG TAC ACC AGG GTC TCC CAG TAC ATC GAG TGG CTG CAA AAG CTC ATG
 Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu Trp Leu Gln Lys Leu Met

1089 1104 1119 1138
 CGC TCA GAG CCA CGC CCA GGA GTC CTC CTG CGA GCC CCA TTT CCC TAG CCCAGCAGCC
 Arg Ser Glu Pro Arg Pro Gly Val Leu Leu Arg Ala Pro Phe Pro

1148 1158 1168 1178 1188 1198 PstIc 1208
 CTGGCCTGTG GAGAGAAAGC CAAGGCTGCG TCGAACTGTC CTGGCACCAA ATCCCATATA TTCTTCTGCA

1218 1228 1238 1248 1258 1268 1278
 GTTAATGGGG TAGAGGAGGG CATGGGAGGG AGGGAGAGGT GGGGAGGGAG ACAGAGACAG AACAGAGAG

1288 1298 1308 1318 1328 1338 1348
 AGACAGAGAC AGAGAGAGAC TGAGGGAGAG ACTCTGAGGA CCATGGACAG AGACTCAAAG AGACTCCAAG

1358 1368 1378 1388 1398 1408 1418
 ATTCAAAGAG ACTAATAGAG ACACAGAGAT GGAATAGAAA AGATGAGAGG CAGAGGCAGA CAGGCGCTGG

1428 1438 1448 1458 1468 1478 1488
 ACAGAGGGGC AGGGGAGTGC CAAGGTTGTC CTGGAGGCAG ACAGCCCAGC TGAGCCTCCT TACCTCCCTT

1498	1508	1518	1528	1538	1548	1558
CAGCCAAGCC	CCACCTGCAC	GTGATCTGCT	GGCCCTCAGG	CTGCTGCTCT	GCCTTCATTG	CTGGAGACAG
1568	1578	1588	1598	1608	1618	1628
TAGAGGCATG	ACACACATGG	ATGCACACAC	ACACACGCCA	TGCACACACA	CAGAGATATG	CACACACACG
1638	1648	1658	1668	1678	1688	1698
GATGCACACA	CAGATGGTCA	CACAGAGTAC	GCAAACACAC	CGATGCACAC	GCACATAGAG	ATATGCACAC
1708	1718	1728	1738	1748	1758	1768
ACAGATGCAC	ACACAGATAT	ACACATGGAG	TGCACGCACA	TGCCAATGCA	CGCACACATC	AGTGCACACG
1778	1788	1798	1808	1818	1828	1838
GATGCACAGA	GATATGCACA	CACCGATGTG	CGCACACACA	GATATGCACA	CACATGGATG	AGCACACACA
1848	1858	1868	1878	1888	1898	1908
CACCAAGTGC	GCACACACAC	CGATGTACAC	ACAGATGCAC	ACACAGATGC	ACACACACCG	ATGCTGACTC
1918	1928	1938	1948	1958	1968	1978
CATGTGTGCT	GTCCTCTGAA	GGCGGTTGTT	TAGCTCTCAC	TTTTCTGGTT	CTTATCCATT	ATCATCTTCA
1988	1998	2008	2018	2028	2038	2048
CTTCAGACAA	TTCAGAAGCA	TCACCATGCA	TGGTGGCGAA	TGCCCCCAAA	CTCTCCCCCA	AATGTATTTC
2058	2068	2078	2088	2098	2108	2118
TCCCTTCGCT	GGGTGCCGGG	CTGCACAGAC	TATTCGCCAC	CTGCTTCCCA	GCTTCACAAT	AAACGGCTGC
2128	2138	2148	2158	2168	EcoRIb	
GTCTCCTCGC	AAAAAAAAAA	AAAAAAAAAA	AAAAAAAAAA	AAAAAAAAAA	<u>AAGGAATTC</u>	

FIG. 1B

-60
 MetValSerGlnAlaLeuArgLeuLeu
 TCAACAGGCAGGGGCAGCACTGCAGAGATTTTCATCATGGTCTCCCAGGCCCTCAGGCTCCTC
 10 20 30 40 50 60

-50 -40 ↓
 CysLeuLeuGlyLeuGlnGlyCysLeuAlaAlaGlyGlyValAlaLysAlaSerGlyGly
 TGCCTTCTGCTTGGGCTTCAGGGCTGCCTGGCTGCAGGCGGGGTCGCTAAGGCCTCAGGAGGA
 70 80 90 100 110 120

-30 -20 ↓
 GluThrArgAspMetProTrpLysProGlyProHisArgValPheValThrGlnGluGlu
 GAAACACGGGACATGCCGTGGAAGCCGGGGCCTCACAGAGTCTTCGTAAACCAGGAGGAA
 130 140 150 160 170 180

-10 -1 +1 +10
 AlaHisGlyValLeuHisArgArgArgArgAlaAsnAlaPheLeuGluGluLeuArgPro
 GCCACGGCGTCTGCACCGGCGCCGGCGGCCAACGCGTTCCTGGAGGAGCTGCGGCCG
 190 200 210 220 230 240

+20 +30
 GlySerLeuGluArgGluCysLysGluGlnCysSerPheGluGluAlaArgGluIle
 GGCTCCCTGGAGAGGGAGTGCAAGGAGGAGCAGTGCTCCTTCGAGGAGGCCCGGGAGATC
 250 260 270 280 290 300

+40 +50
 PheLysAspAlaGluArgThrLysLeuPheTrpIleSerTyrSerAspGlyAspGlnCys
 TTCAAGGACGCGGAGAGGACGAAGCTGTTCTGGATTTCTTACAGTGATGGGGACCAAGTGT
 310 320 330 340 350 360

+60 +70
 AlaSerSerProCysGlnAsnGlyGlySerCysLysAspGlnLeuGlnSerTyrIleCys
 GCCTCAAGTCCATGCCAGAATGGGGGCTCCTGCAAGGACCAGCTCCAGTCTATATCTGC
 370 380 390 400 410 420

+80 +90
 PheCysLeuProAlaPheGluGlyArgAsnCysGluThrHisLysAspAspGlnLeuIle
 TTCTGCCTCCCTGCCTTCGAGGGCCGGAAGTGTGAGACGCACAAGGATGACCAGCTGATC
 430 440 450 460 470 480

+100 +110
 CysValAsnGluAsnGlyGlyCysGluGlnTyrCysSerAspHisThrGlyThrLysArg
 TGTGTGAACGAGAACGGCGGCTGTGAGCAGTACTGCAGTGACCACACGGGCACCAAGCGC
 490 500 510 520 530 540

+120 +130
 SerCysArgCysHisGluGlyTyrSerLeuLeuAlaAspGlyValSerCysThrProThr
 TCCTGTCTGGTGCCACGAGGGGTACTCTCTGCTGGCAGACGGGGTGTCTCTGCACACCCACA
 550 560 570 580 590 600

+140 +150
 ValGluTyrProCysGlyLysIleProIleLeuGluLysArgAsnAlaSerLysProGln
 GTTGAATATCCATGTGGAAAAATACCTATTCTAGAAAAAAGAAATGCCAGCAAACCCCAA
 610 620 630 640 650 660

		+160		+170
GlyArgIleValGlyGlyLysValCysProLysGlyGluCysProTrpGlnValLeuLeu				
GGCCGAATTGTGGGGGGCAAGGTGTGCCCCAAAGGGGAGTGTCCATGGCAGGTCTGTGTG				
670	680	690	700	710 720

		+180		+190
LeuValAsnGlyAlaGlnLeuCysGlyGlyThrLeuIleAsnThrIleTrpValValSer				
TTGGTGAATGGAGCTCAGTTGTGTGGGGGGACCCTGATCAACACCATCTGGGTGGTCTCC				
730	740	750	760	770 780

		+200		+210
AlaAlaHisCysPheAspLysIleLysAsnTrpArgAsnLeuIleAlaValLeuGlyGlu				
GCGGCCCACTGTTTCGACAAAATCAAGAACTGGAGGAACCTGATCGCGGTGCTGGGCGAG				
790	880	810	820	830 840

		+220		+230
HisAspLeuSerGluHisAspGlyAspGluGlnSerArgArgValAlaGlnValIleIle				
CACGACCTCAGCGAGCACGACGGGGATGAGCAGAGCCGGCGGGTGGCGCAGGTCATCATC				
850	860	870	880	890 900

		+240		+250
ProSerThrTyrValProGlyThrThrAsnHisAspIleAlaLeuLeuArgLeuHisGln				
CCCAGCACGTACGTCCCGGGCACCACCAACCACGACATCGCGCTGCTCCGCCTGCACCAG				
910	920	930	940	950 960

		+260		+270
ProValValLeuThrAspHisValValProLeuCysLeuProGluArgThrPheSerGlu				
CCCGTGGTCCTCACTGACCATGTGGTGCCCTCTGCCTGCCCCAACGGACGTTCTCTGAG				
970	980	990	1000	1010 1020

		+280		+290
ArgThrLeuAlaPheValArgPheSerLeuValSerGlyTrpGlyGlnLeuLeuAspArg				
AGGACGCTGGCCTTCGTGCGCTTCTCATTGGTCAGCGGCTGGGGCCAGCTGCTGGACCGT				
1030	1040	1050	1060	1070 1080

		+300		+310
GlyAlaThrAlaLeuGluLeuMetValLeuAsnValProArgLeuMetThrGlnAspCys				
GGCGCCACGGCCCTGGAGCTCATGGTCCTCAACGTGCCCCGGCTGATGACCCAGGACTGC				
1090	1100	1110	1120	1130 1140

		+320		+330
LeuGlnGlnSerArgLysValGlyAspSerProAsnIleThrGluTyrMetPheCysAla				
CTGCAGCAGTCACGGAAGGTGGGAGACTCCCCAAATATCACGGAGTACATGTTCTGTGCC				
1150	1160	1170	1180	1190 1200

		+340		+350
GlyTyrSerAspGlySerLysAspSerCysLysGlyAspSerGlyGlyProHisAlaThr				
GGCTACTCGGATGGCAGCAAGGACTCCTGCAAGGGGGACAGTGGAGGCCACATGCCACC				
1210	1220	1230	1240	1250 1260

		+360		+370
HisTyrArgGlyThrTrpTyrLeuThrGlyIleValSerTrpGlyGlnGlyCysAlaThr				
CACTACCGGGGCACGTGGTACCTGACGGGCATCGTCAGCTGGGGCCAGGGCTGCGCAACC				
1270	1280	1290	1300	1310 1320

						+380							+390						
Val	Gly	His	Phe	Gly	Val	Tyr	Thr	Arg	Val	Ser	Gln	Tyr	Ile	Glu	Trp	Leu	Gln	Lys	Leu
GTGGGCCACTTTGGGGTGTACACCAGGGTCTCCAGTACATCGAGTGGCTGCAAAAGCTC																			
1330		1340		1350		1360		1370		1380									
						+400							+406						
Met	Arg	Ser	Glu	Pro	Arg	Pro	Gly	Val	Leu	Leu	Arg	Ala	Pro	Phe	Pro	***			
ATGCGCTCAGAGCCACGCCCAGGAGTCCTCCTGCGAGCCCCATTTCCCTAGCCCAGCAGC																			
1390		1400		1410		1420		1430		1440									
CCTGGCCTGTGGAGAGAAAGCCAAGGCTGCGTCGAACTGTCTGGCACCAAATCCCATAT																			
1450		1460		1470		1480		1490		1500									
ATTCTTCTGCAGTTAATGGGGTAGAGGAGGGCATGGGAGGGAGGGAGAGGTGGGGAGGGA																			
1510		1520		1530		1540		1550		1560									
GACAGAGACAGAAACAGAGAGAGACAGAGACAGAGAGAGACTGAGGGAGAGACTCTGAGG																			
1570		1580		1590		1600		1610		1620									
ACATGGAGAGAGACTCAAAGAGACTCCAAGATTCAAAGAGACTAATAGAGACACAGAGAT																			
1630		1640		1650		1660		1670		1680									
GGAATAGAAAAGATGAGAGGCAGAGGCAGACAGGCGCTGGACAGAGGGGCAGGGGAGTGC																			
1690		1700		1710		1720		1730		1740									
CAAGGTTGTCTGGAGGCAGACAGCCCAGCTGAGCCTCCTTACCTCCCTTCAGCCAAGCC																			
1750		1760		1770		1780		1790		1800									
CCACCTGCACGTGATCTGCTGGCCCTCAGGCTGCTGCTCTGCCTTCATTGCTGGAGACAG																			
1810		1820		1830		1840		1850		1860									
TAGAGGCATGAACACACATGGATGCACACACACACACGCCAATGCACACACACAGAGATA																			
1870		1880		1890		1900		1910		1920									
TGCACACACACGGATGCACACACAGATGGTCAACAGAGATACGCAAACACACCGATGCA																			
1930		1940		1950		1960		1970		1980									
CACGCACATAGAGATATGCACACACAGATGCACACACAGATATACACATGGATGCACGCA																			
1990		2000		2010		2020		2030		2040									
CATGCCAATGCACGCACACATCAGTGCACACGGATGCACAGAGATATGCACACACCGATG																			
2050		2060		2070		2080		2090		2100									
TGCGCACACACAGATATGCACACACATGGATGAGCACACACACACCAAGTGCGCACACAC																			
2110		2120		2130		2140		2150		2160									
ACCGATGTACACACACAGATGCACACACAGATGCACACACACCGATGCTGACTCCATGTG																			
2170		2180		2190		2200		2210		2220									
TGCTGTCCTCTGAAGGCGGTTGTTTAGCTCTCACTTTTCTGGTTCTTATCCATTATCATC																			
2230		2240		2250		2260		2270		2280									
TTCCTTCAGACAATTCAGAAGCATCACCATGCATGGTGGCGAATGCCCCAAACTCTCC																			
2290		2300		2310		2320		2330		2340									
CCCAAATGTATTTCTCCCTTCGCTGGGTGCCGGGCTGCACAGACTATTCCCCACCTGCTT																			
2350		2360		2370		2380		2390		2400									

CCCAGCTTCACAATAAACGGCTGCGTCTCCTCCGCACACCTGTGGTGCCTGCCACCCAAA
2410 2420 2430 2240 2450 2460

AAAAAAAAAAAAAAAAAAAA
2470 2480

1 10 20 30 40
 * * * * * * * *
 FACTOR VII A N A - F L Y Y L R P G S L Y R Y C K Y Y Q C S F Y Y A R Y I F Y X X X R I K L
 FACTOR IX Y N S G K L Y Y F V Q G N L Y R Y C H Y Y K C S F Y Y A R Y V F Y N T Y R T T Y
 FACTOR X A N S - F L Y Y M K K G H L Y R Y C H Y Y T C S Y Y A R Y V F Y D S D K T M Y
 PROTEIN C A N S - F L Y Y L R H S S L Y R Y C I Y Y I C D F Y Y A L Y I F Q N V D D I L A
 PROTHROMBIN A N T - F L Y Y V R K G N L Y R Y C V Y Y T C S Y Y A F Y A L Y S S T A T D V

 50 60 70
 F W I S Y S D G D Q C A S S - - - - - P C Q N G G S C K D Q L Q S Y I C F
 FACTOR VII F W K Q Y V D G D Q C E S N - - - - - P C L N G G S C K B D I N S Y E C W
 FACTOR IX F W N K Y K D G D Q C E T S - - - - - P C Q N Q G K C K B G L C E Y T C T
 FACTOR X F W S K H V D G D Q C L V L P L E H P C A S L C C G H G T C I B G I G S F S C D
 PROTEIN C F W A K Y I A C E T A R T P R D K L A A C L E G N C A E G L C T N Y R C H V N I
 PROTHROMBIN

 80 90 100 110
 C L P A P E G R N C E T H K D D Q L I C V N E N G G C E Q Y C S D H T G T K R S C
 FACTOR VII C P F G F E G K N C E L D V T - - - - - C N I K N C R C E Q F C K N S A D N K V V C
 FACTOR IX C L E G F E G K N C E L F T R K L - - - - - C S L D N G D C D Q F C H E E Q N S - V V C
 FACTOR X C R S G W E G R F C Q R E V S F L N - C S L D N G G C T H Y C L E E V G H - R R C
 PROTEIN C T R S G I E C Q L W R S R Y P H K P - E I N S T T H P G A D L Q E N F C R N P D S
 PROTHROMBIN

 120 130 140
 R C H E G Y S L L A D G V S C T P T V E Y P C G K I P I L E K R N A S K P Q G R
 FACTOR VII S C T E G Y R L A G N Q K S C E P A V P F P C G R V S V S Q T S K L R T
 FACTOR IX S C A R G Y T L A D N G K A C I P T G P Y P C G K Q T L E R
 FACTOR X S C A P G Y K L G D D L L Q C H P A V K P F C G R P W K R N E K R S H L
 PROTEIN C S N T G P W C Y T T D P T V R R Q E C S I P V C G Q D Q V T V A M T P R S
 PROTHROMBIN

FIG.
2A

[illegible]

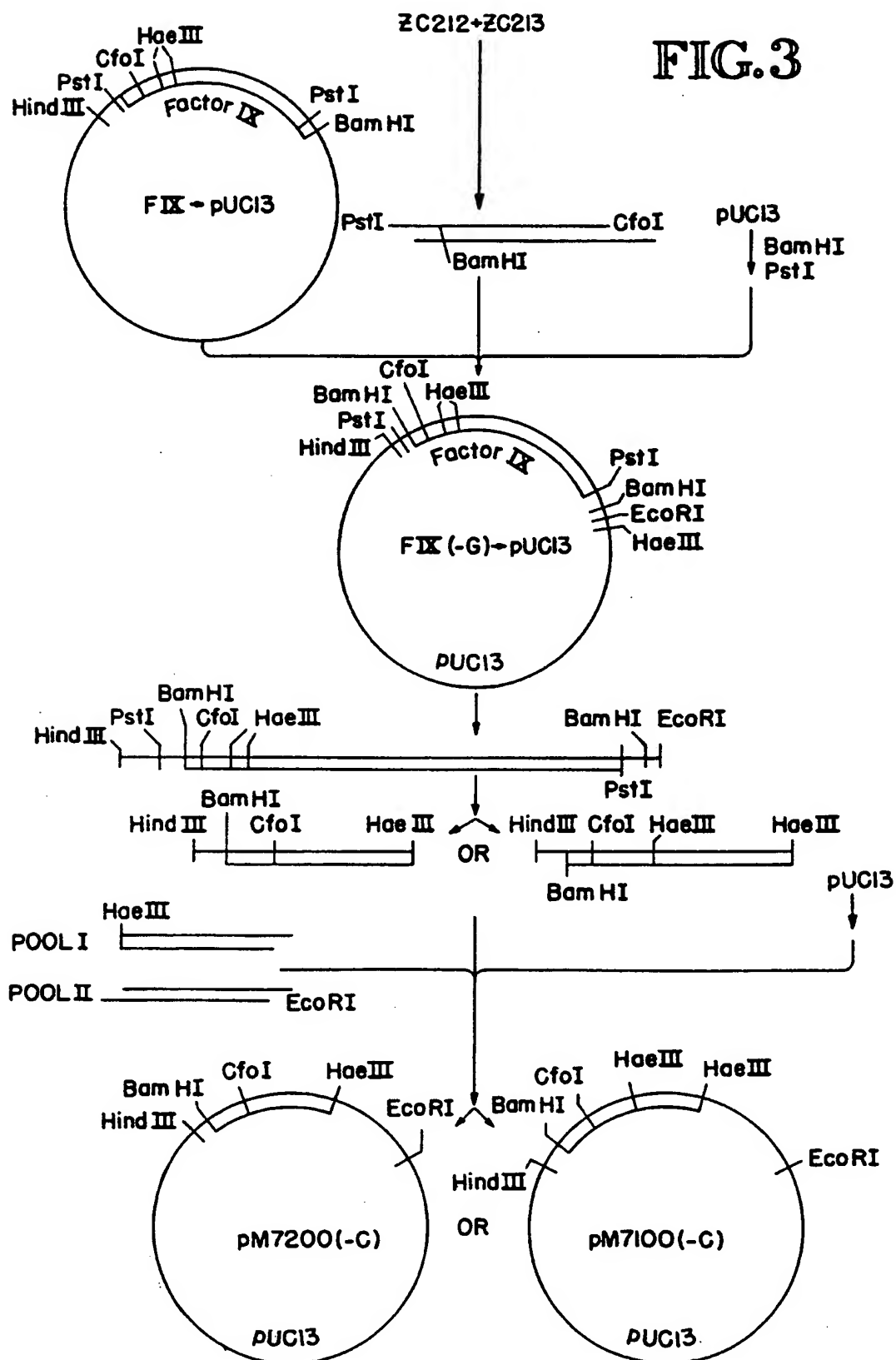
	40	50	60	70
cDNA	K L F W I S Y S D G D Q C A S S P C N G G S C K D Q L Q S Y I C P C L			
Amino Acid Sequence	L F W I S Y S D G D Q C A S S P C N G G S C K D Q L Q I C P C L			

		80	90	100	
cDNA	P A F E G R N C E T H K D D Q L I C V N E N G G C E Q Y C S D H T G T K				
Amino Acid Sequence	P A F E G R N C E T H K D D Q L C S D H T G T				

	110	120	130	140
cDNA	R S C R C H E G Y S L L A D G V S C T P T V E Y P C G K I P I L E K R N			
Amino Acid Sequence	S C R C H E G Y S L L A D G V S C T P T V E Y			E K R ()

	150	
cDNA	A S K P Q G R	
Amino Acid Sequence	A S K P Q G R	

FIG. 3



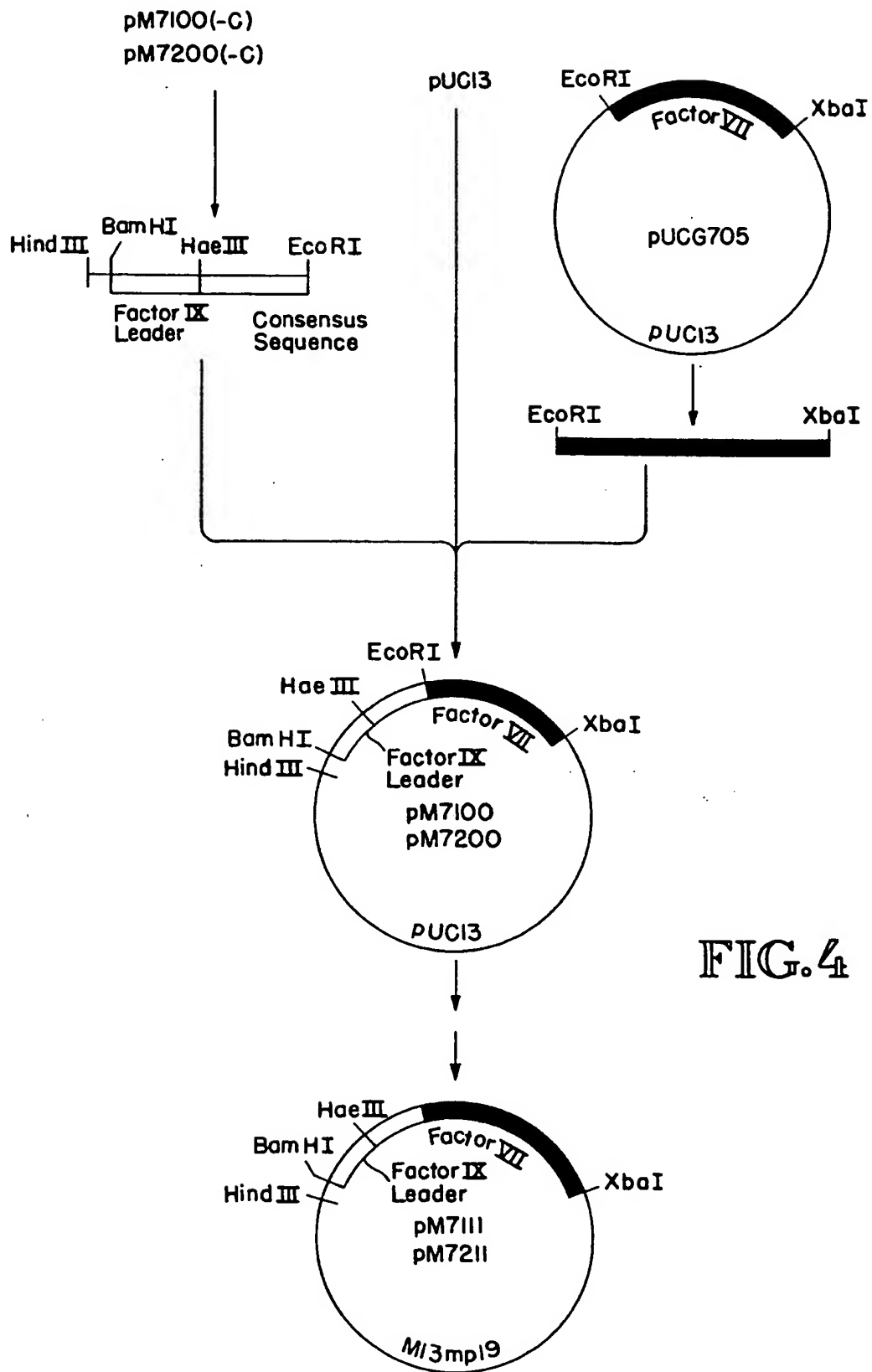


FIG.4

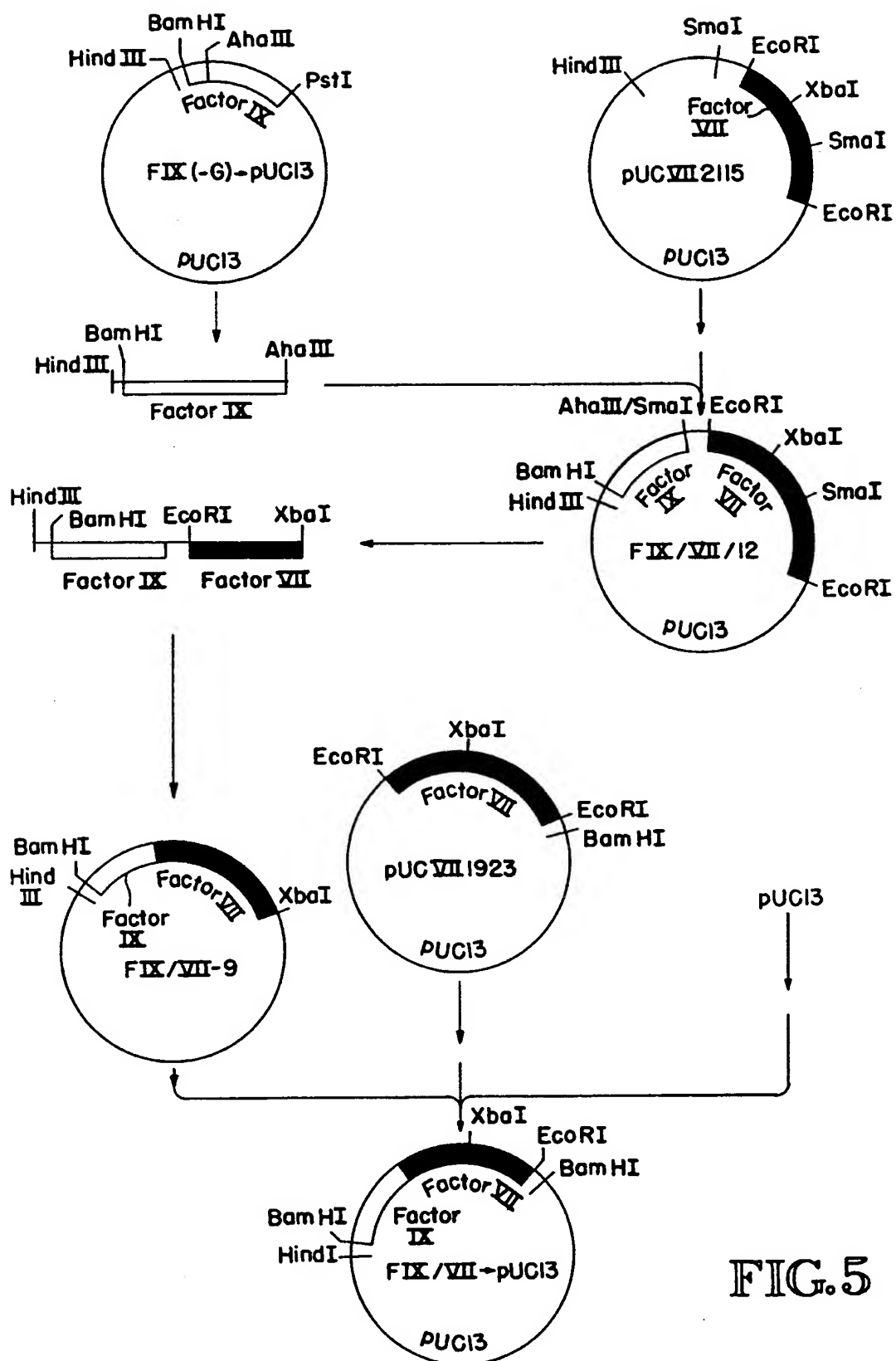


FIG.5

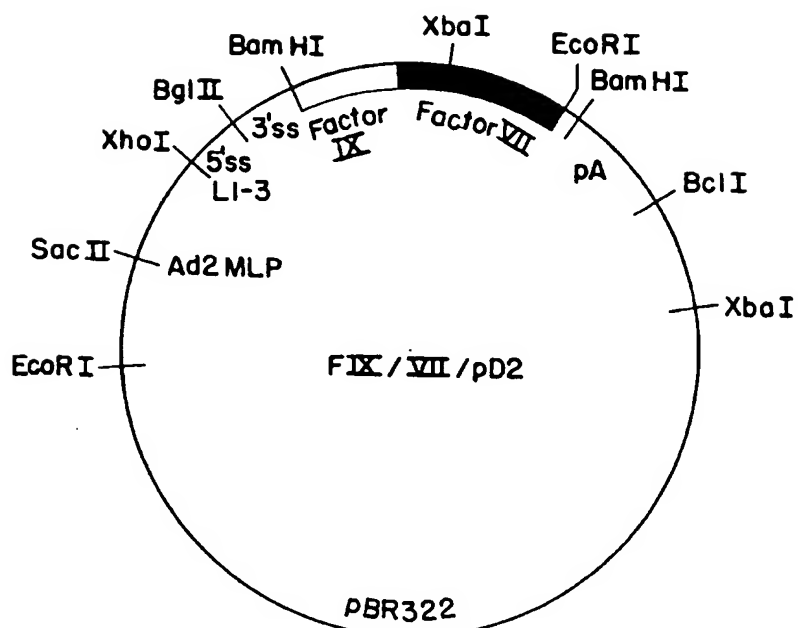


FIG. 6

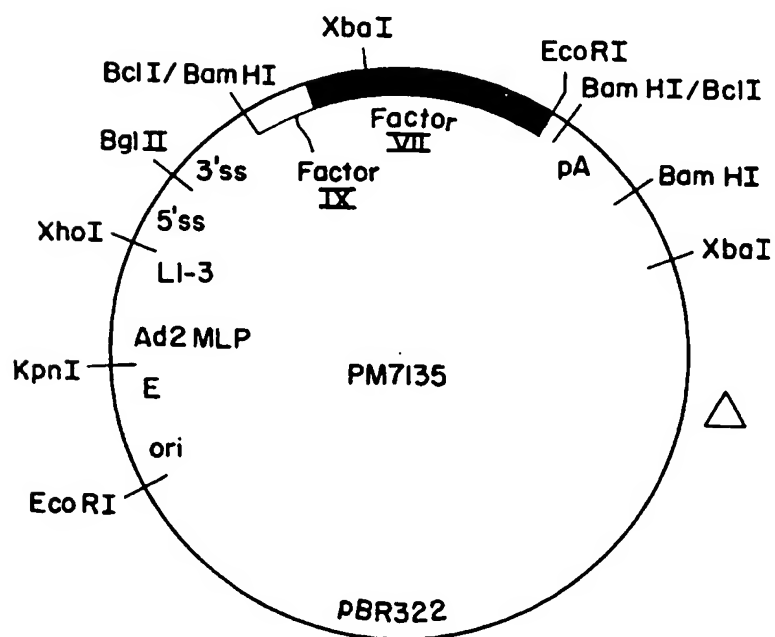


FIG. 8

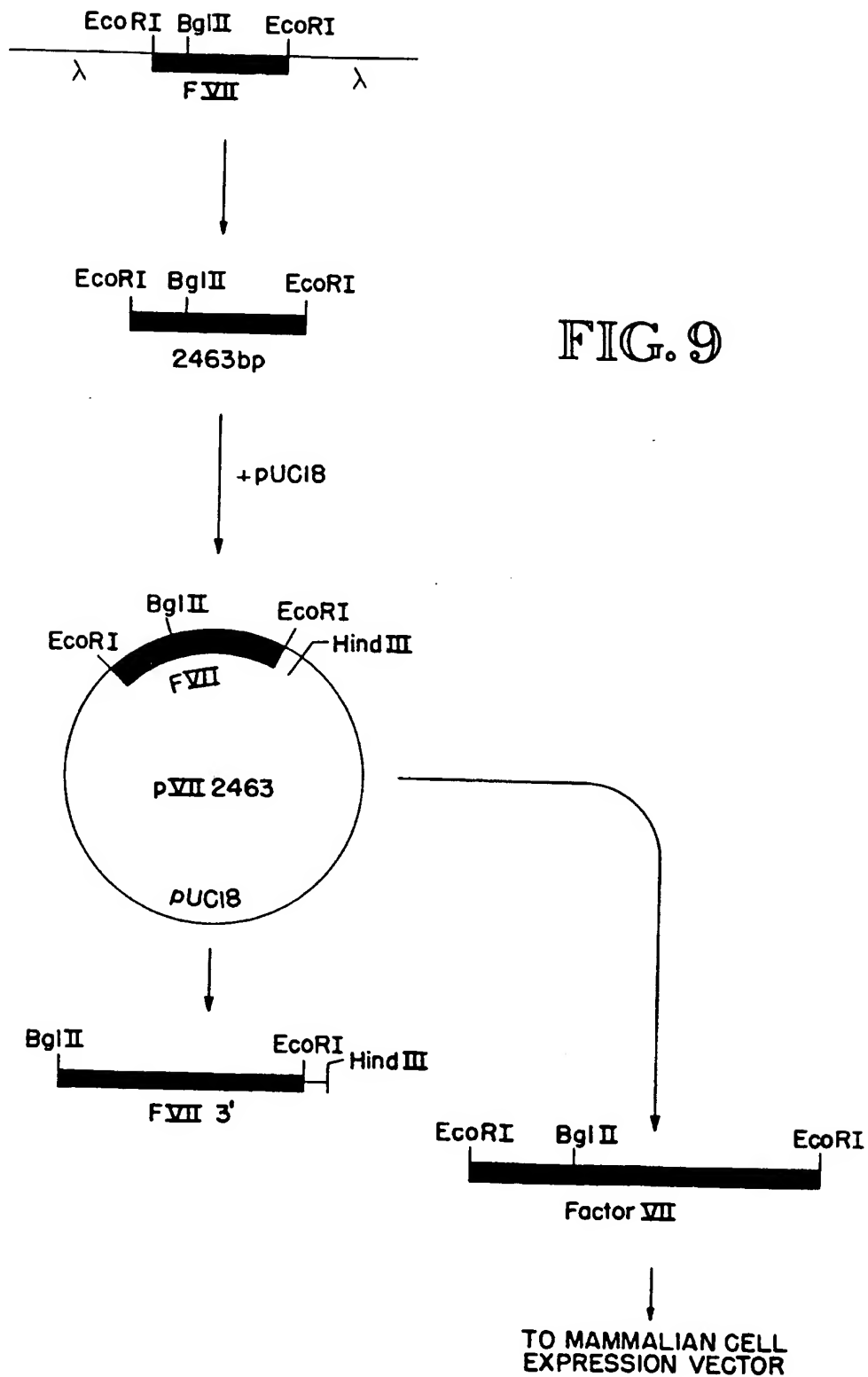
FIG. 7

												21													36													
GGATCC	ATG	CAG	CGC	GTG	AAC	ATG	ATC	ATG	GCA	GAA	TCA	CCA	GGC																									
												MET	Gln	Arg	Val	Asn	MET	Ile	MET	Ala	Glu	Ser	Pro	Gly														
												66													81													
CTC	ATC	ACC	ATC	TGC	CTT	TTA	GGA	TAT	CTA	CTC	AGT	GCT	GAA	TGT																								
Leu	Ile	Thr	Ile	Cys	Leu	Leu	Gly	Tyr	Leu	Leu	Ser	Ala	Glu	Cys																								
												96													111													126
ACA	GTT	TTT	CTT	GAT	CAT	GAA	AAC	GCC	AAC	AAA	ATT	CTG	AAT	CGG																								
Thr	Val	Phe	Leu	Asp	His	Glu	Asn	Ala	Asn	Lys	Ile	Leu	Asn	Arg																								
												141													156													171
CCA	AAG	AGG	TAT	AAT	TCA	GGT	AAA	TTG	GAA	GAG	TTT	GTT	CAA	GGG																								
Pro	Lys	Arg	Tyr	Asn	Ser	Gly	Lys	Leu	Glu	Glu	Phe	Val	Gln	Gly																								
												186													201													216
AAC	CTT	GAG	AGA	GAA	TGT	ATG	GAA	GAA	AAG	TGT	AGT	TTT	GAA	GAA																								
Asn	Leu	Glu	Arg	Glu	Cys	MET	Glu	Glu	Lys	Cys	Ser	Phe	Glu	Glu																								
												231													246													261
GCA	CGA	GAA	GTT	TTT	GAA	AAC	ACT	GAA	AGA	ACA	AAG	CTG	TTC	TGG																								
Ala	Arg	Glu	Val	Phe	Glu	Asn	Thr	Glu	Arg	Thr	Lys	Leu	Phe	Trp																								
												276													291													306
ATT	TCT	TAC	AGT	GAT	GGG	GAC	CAG	TGT	GCC	TCA	AGT	CCA	TGC	CAG																								
Ile	Ser	Tyr	Ser	Asp	Gly	Asp	Gln	Cys	Ala	Ser	Ser	Pro	Cys	Gln																								
												321													336													351
AAT	GGG	GGC	TCC	TGC	AAG	GAC	CAG	CTC	CAG	TCC	TAT	ATC	TGC	TTC																								
Asn	Gly	Gly	Ser	Cys	Lys	Asp	Gln	Leu	Gln	Ser	Tyr	Ile	Cys	Phe																								
												366													381													396
TGC	CTC	CCT	GCC	TTC	GAG	GGC	CGG	AAC	TGT	GAG	ACG	CAC	AAG	GAT																								
Cys	Leu	Pro	Ala	Phe	Glu	Gly	Arg	Asn	Cys	Glu	Thr	His	Lys	Asp																								
												411													426													441
GAC	CAG	CTG	ATC	TGT	GTG	AAC	GAG	AAC	GGC	GGC	TGT	GAG	CAG	TAC																								
Asp	Glu	Leu	Ile	Cys	Val	Asn	Glu	Asn	Gly	Gly	Cys	Glu	Gln	Tyr																								
												456													471													486
TGC	AGT	GAC	CAC	ACG	GGC	ACC	AAG	CGC	TCC	TGT	CGG	TGC	CAC	GAG																								
Cys	Ser	Asp	His	Thr	Gly	Thr	Lys	Arg	Ser	Cys	Arg	Cys	His	Glu																								
												501													516													531
GGG	TAC	TCT	CTG	CTG	GCA	GAC	GGG	GTG	TCC	TGC	ACA	CCC	ACA	GTT																								
Gly	Tyr	Ser	Leu	Leu	Ala	Asp	Gly	Val	Ser	Cys	Thr	Pro	Thr	Val																								
												546													561													576
GAA	TAT	CCA	TCT	GGA	AAA	ATA	CCT	ATT	CTA	GAA	AAA	AGA	AAT	GCC																								
Glu	Tyr	Pro	Cys	Gly	Lys	Ile	Pro	Ile	Leu	Glu	Lys	Arg	Asn	Ala																								
												591													606													621
AGC	AAA	CCC	CAA	GGC	CGA	ATT	GTG	GGG	GGC	AAG	GTG	TGC	CCC	AAA																								
Ser	Lys	Pro	Gln	Gly	Arg	Ile	Val	Gly	Gly	Lys	Val	Cys	Pro	Lys																								

636	GGG	GAG	TGT	CCA	TGG	CAG	GTC	CTG	TTG	TTG	GTG	AAT	GGA	GCT	CAG
	Gly	Glu	Cys	Pro	Trp	Gln	Val	Leu	Leu	Leu	Val	Asn	Gly	Ala	Gln
681	TTG	TGT	GGG	GGG	ACC	CTG	ATC	AAC	ACC	ATC	TGG	GTG	GTC	TCC	GCG
	Leu	Cys	Gly	Gly	Thr	Leu	Ile	Asn	Thr	Ile	Trp	Val	Val	Ser	Ala
726	GCC	CAC	TGT	TTC	GAC	AAA	ATC	AAG	AAC	TGG	AGG	AAC	CTG	ATC	GCG
	Ala	His	Cys	Phe	Asp	Lys	Ile	Lys	Asn	Trp	Arg	Asn	Leu	Ile	Ala
771	GTG	CTG	GGC	GAG	CAC	GAC	CTC	AGC	GAG	CAC	GAC	GGG	GAT	GAG	CAG
	Val	Leu	Gly	Glu	His	Asp	Leu	Ser	Glu	His	Asp	Gly	Asp	Glu	Gln
816	AGC	CGG	CGG	GTG	GCG	CAG	GTC	ATC	ATC	CCC	AGC	ACG	TAC	GTC	CCG
	Ser	Arg	Arg	Val	Ala	Gln	Val	Ile	Ile	Pro	Ser	Thr	Tyr	Val	Pro
861	GGC	ACC	ACC	AAC	CAC	GAC	ATC	GCG	CTG	CTC	CGC	CTG	CAC	CAG	CCC
	Gly	Thr	Thr	Asn	His	Asp	Ile	Ala	Leu	Leu	Arg	Leu	His	Gln	Pro
906	GTG	GTC	CTC	ACT	GAC	CAT	GTG	GTG	CCC	CTC	TGC	CTG	CCC	GAA	CGG
	Val	Val	Leu	Thr	Asp	His	Val	Val	Pro	Leu	Cys	Leu	Pro	Glu	Arg
951	ACG	TTC	TCT	GAG	AGG	ACG	CTG	GCC	TTC	GTG	CGC	TTC	TCA	TTG	GTC
	Thr	Phe	Ser	Glu	Arg	Thr	Leu	Ala	Phe	Val	Arg	Phe	Ser	Leu	Val
996	AGC	GGC	TGG	GGC	CAG	CTG	CTG	GAC	CGT	GGC	GCC	ACG	GCC	CTG	GAG
	Ser	Gly	Trp	Gly	Gln	Leu	Leu	Asp	Arg	Gly	Ala	Thr	Ala	Leu	Glu
1041	CTC	ATG	GTC	CTC	AAC	GTG	CCC	CGG	CTG	ATG	ACC	CAG	GAC	TGC	CTG
	Leu	MET	Val	Leu	Asn	Val	Pro	Arg	Leu	MET	Thr	Gln	Asp	Cys	Leu
1086	CAG	CAG	TCA	CGG	AAG	GTG	GGA	GAC	TCC	CCA	AAT	ATC	ACG	GAG	TAC
	Gln	Gln	Ser	Arg	Lys	Val	Gly	Asp	Ser	Pro	Asn	Ile	Thr	Glu	Tyr
1131	ATG	TTC	TGT	GCC	GGC	TAC	TCG	GAT	GGC	AGC	AAG	GAC	TCC	TGC	AAG
	MET	Phe	Cys	Ala	Gly	Tyr	Ser	Asp	Gly	Ser	Lys	Asp	Ser	Cys	Lys
1176	GGG	GAC	AGT	GGA	GGC	CCA	CAT	GCC	ACC	CAC	TAC	CGG	GGC	ACG	TGG
	Gly	Asp	Ser	Gly	Gly	Pro	His	Ala	Thr	His	Tyr	Arg	Gly	Thr	Trp
1221	TAC	CTG	ACG	GGC	ATC	GTC	AGC	TGG	GGC	CAG	GGC	TGC	GCA	ACC	GTG
	Tyr	Leu	Thr	gly	Ile	Val	Ser	Trp	Gly	Gln	Gly	Cys	Ala	Thr	Val

1266	1281	1296		
GGC CAC TTT GGG GTG TAC ACC AGG GTC TCC CAG TAC ATC GAG TGG				
Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu Trp				
1311	1326	1341		
CTG CAA AAG CTC ATG CGC TCA GAG CCA CGC CCA GGA GTC CTC CTG				
Leu Gln Lys Leu MET Arg Ser Glu Pro Arg Pro Gly Val Leu Leu				
1356	1378	1388	1398	
CGA GCC CCA TTT CCC TAG CCCAGCAGCC CTGGCCTGTG GAGAGAAAGC				
Arg Ala Pro Phe Pro				
1408	1418	1428	1438	1448
CAAGGCTGCG TCGAACTGTC CTGGCACCAA ATCCCATATA TTCTTCTGCA				
1458	1468	1478	1488	1498
GTTAATGGGG TAGAGGAGGG CATGGGAGGG AGGGAGAGGT GGGGAGGGAG				
1508	1518	1528	1538	1548
ACAGAGACAG AAACAGAGAG AGACAGAGAC AGAGAGAGAC TGAGGGAGAG				
1558	1568	1578	1588	1598
ACTCTGAGGA CCATGGAGAG AGACTCAAAG AGACTCCAAG ATTCAAAGAG				
1608	1618	1628	1638	1648
ACTAATAGAG ACACAGAGAT GGAATAGAAA AGATGAGAGG CAGAGGCAGA				
1658	1668	1678	1688	1698
CAGGCGCTGG ACAGAGGGGC AGGGGAGTGC CAAGGTTGTC CTGGAGGCAG				
1708	1718	1728	1738	1748
ACAGCCCAGC TGAGCCTCCT TACCTCCCTT CAGCCAAGCC CCACCTGCAC				
1758	1768	1778	1788	1798
GTGATCTGCT GGCCCTCAGG CTGCTGCTCT GCCTTCATTG CTGGAGACAG				
1808	1818	1828	1838	1848
TAGAGGCATG ACACACATGG ATGCACACAC ACACACGCCA TGCACACACA				
1858	1868	1878	1888	1898
CAGAGATATG CACACACACG GATGCACACA CAGATGGTCA CACAGAGTAC				
1908	1918	1928	1938	1948
GCAAACACAC CGATGCACAC GCACATAGAG ATATGCACAC ACAGATGCAC				

1958	1968	1978	1988	1998
ACACAGATAT	ACACATGGAG	TGCACGCACA	TGCCAATGCA	CGCACACATC
2008	2018	2028	2038	2048
AGTGACACG	GATGCACAGA	GATATGCACA	CACCGATGTG	CGCACACACA
2058	2068	2078	2088	2098
GATATGCACA	CACATGGATG	AGCACACACA	CACCAAGTGC	GCACACACAC
2108	2118	2128	2138	2148
CGATGTACAC	ACAGATGCAC	ACACAGATGC	ACACACACCG	ATGCTGACTC
2158	2168	2178	2188	2198
CATGTGTGCT	GTCCTCTGAA	GGCGGTTGTT	TAGCTCTCAC	TTTTCTGGTT
2208	2218	2228	2238	2248
CTTATCCATT	ATCATCTTCA	CTTCAGACAA	TTCAGAAGCA	TCACCATGCA
2258	2268	2278	2288	2298
TGGTGCGCAA	TGCCCCCAA	CTCTCCCCCA	AATGTATTTC	TCCCTTCGCT
2308	2318	2328	2338	2348
GGGTGCCGGG	CTGCACAGAC	TATTCCCCAC	CTGCTTCCCA	GCTTCACAAT
2358	2368	2378	2388	2398
AAACGGCTGC	GTCTCCTCGC	AAAAAAAAAA	AAAAAAAAAA	AAAAAAAAAA
2408	2418	2428	2438	
AAAAAAAAAA	AAGGAATTCG	AGCTCGGTAC	CCGGGGATCC	



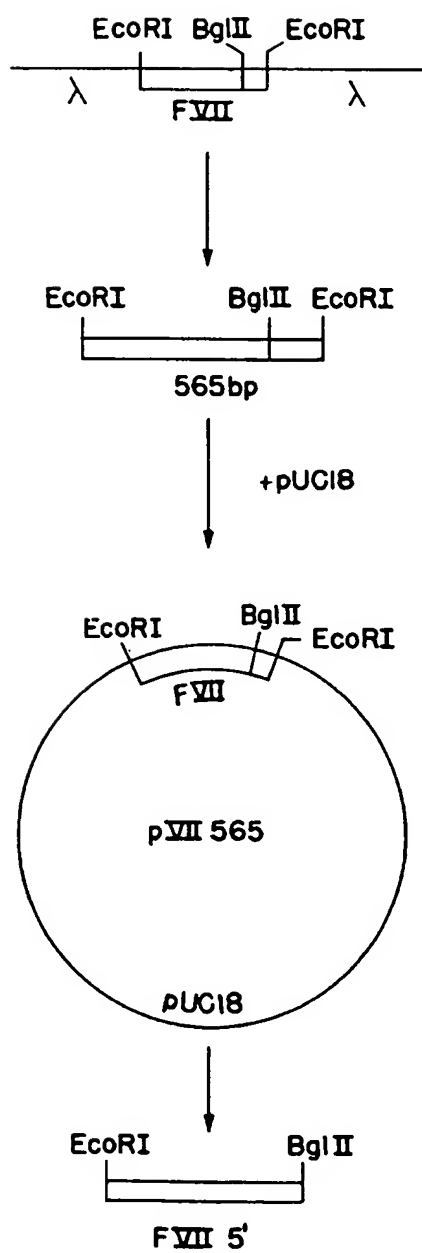


FIG. 10

FIG. 11

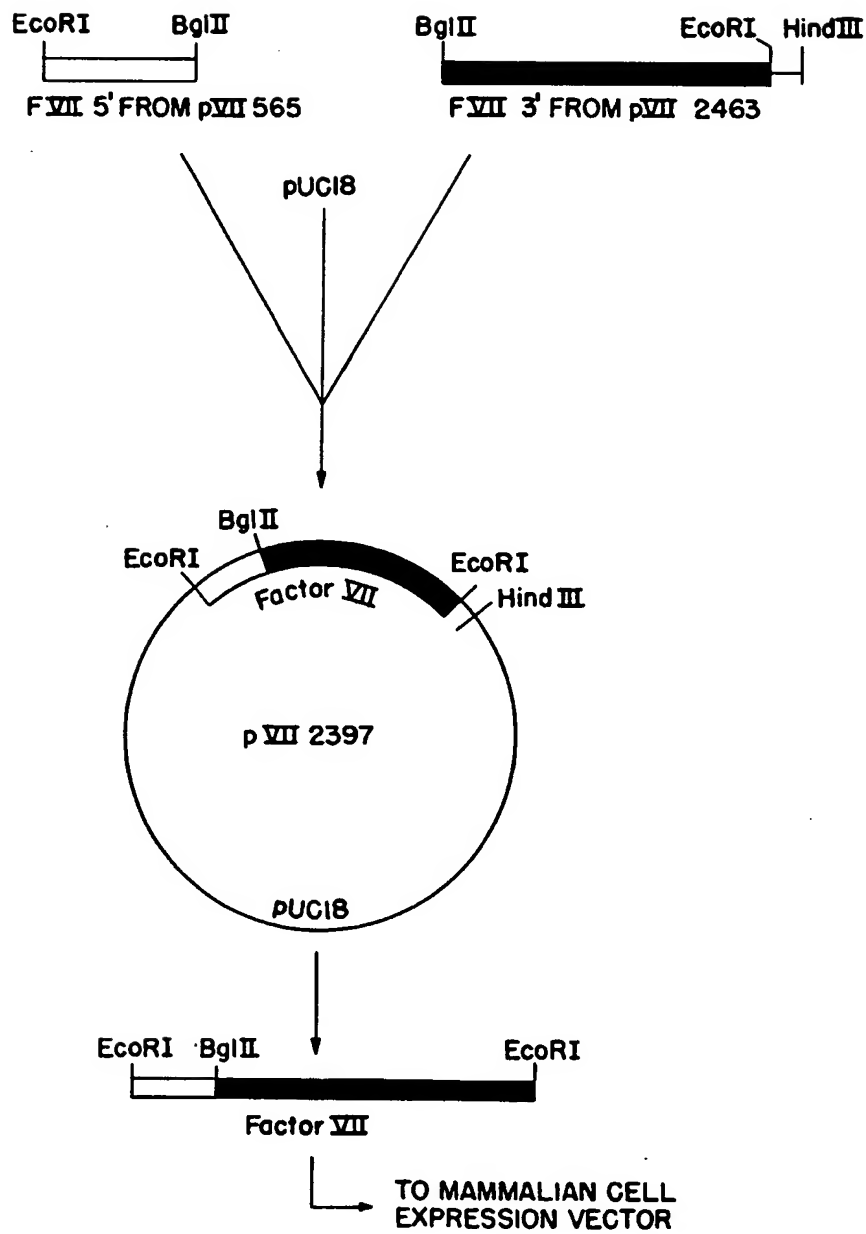


FIG. 12

